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Cancer Cells by Sphingosine Kinase

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13. ABSTRACT (Maximum 200 Words) <p>The sphingolipid metabolite, sphingosine-1-phosphate (S1P) formed by phosphorylation of sphingosine, has been implicated in cell growth, suppression of apoptosis, and angiogenesis. We have examined whether increased intracellular S1P produced by overexpression of sphingosine kinase contributes to tumorigenesis of breast adenocarcinoma MCF-7 cells. Sphingosine kinase type 1 (SPHK1) blocks MCF-7 cell death induced by anti-cancer drugs, sphingosine and TNF-α. The cytoprotective effect of SPHK1 was reversed by N, N'-dimethylsphingosine, a specific inhibitor of SPHKs. Enforced expression of SPHK1 in MCF-7 cells also conferred a growth advantage as determined by proliferation and growth in soft agar. Although no changes in estrogen receptor levels could be detected, estrogen antagonists eliminated this growth advantage. EDG-1 is a GPCR for S1P that mediates S1P-directed cell migration and vascular maturation. Cell migration toward PDGF, which stimulates sphingosine kinase and increases S1P levels, was dependent on expression of S1P1/EDG-1, and conversely, deletion of S1P1/EDG-1, inhibition of sphingosine kinase or treatment with pertussis toxin to uncouple Gi-linked receptors suppressed chemotaxis toward PDGF. PDGF induced tyrosine phosphorylation of focal adhesion proteins, including FAK and Src whereas tyrosine phosphorylation of the PDGFR and ERK activation was unaltered. These results suggest SPHK as a possible target for cancer therapy since it seems to be involved in cell migration and survival.</p>				
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INTRODUCTION

The bioactive sphingolipid metabolite, sphingosine-1-phosphate (S1P), has emerged as a novel lipid second messenger involved in the regulation of proliferation, survival, and motility of many cell types. S1P is produced after phosphorylation of sphingosine by sphingosine kinase, and has been shown to prevent apoptosis induced by elevated levels of ceramide. Interest in S1P has accelerated recently with the discovery that it is the extracellular ligand for S1P1/EDG-1, S1P3/-3, S1P2/-5, S1P4/-6, and S1P5/-8. Although the biological functions of these receptors have not been completely elucidated, we have recently implicated EDG-1 in cell migration[1] and angiogenesis[2].

Angiogenesis, the process of new vessel formation from pre-existing ones, is essential throughout life, particularly during embryogenesis and development, and is also important in certain pathological conditions such as tumor growth, invasion and metastasis. Recently, In collaboration with Dr. Proia's lab from the NIH we found that disruption of the *s1p1/edg-1* gene by homologous recombination in mice resulted in massive intra-embryonic hemorrhaging and intrauterine death between E12.5 and E14.5 due to incomplete vascular maturation[2]. This defect resulted from a failure of mural cells - vascular smooth muscle cells and pericytes - to migrate to arteries and capillaries and properly reinforce them, resulting in blood vessels composed of mainly naked endothelial tubes. Interestingly, disruption of the PDGF-BB or PDGFR-b genes in mice resulted in a similar lethal phenotype. Since in many different cell types, PDGF stimulates sphingosine kinase leading to an increase in S1P levels, we hypothesized that one of the critical mechanisms in PDGF-induced migration is transactivation of S1P1/EDG-1, and that S1P1/EDG-1 is necessary for PDGF-induced cell motility.

SPHK is activated by numerous external stimuli, among which growth and survival factors are prominent [3]. Enforced expression of SPHK1 in NIH 3T3 fibroblasts increased the proportion of cells in S phase of the cell cycle by promoting the G₁-S transition; it also reduced the doubling time of these cells, an effect that was especially marked under low-serum conditions, indicating that intracellular S1P may be an important regulator of cell growth[4]. Furthermore, SPHK1 expression also resulted in acquisition of a transformed phenotype, as determined by assays of focus formation, colony growth in soft agar, and the ability to form tumors in NOD/SCID mice, suggesting that the corresponding wild-type gene may act as an oncogene [5]. With the use of a SPHK inhibitor and a dominant negative mutant of this enzyme, Xia *et al.* also showed that SPHK contributes to cell transformation mediated by oncogenic H-Ras.

Overexpression of SPHK1 also protected against apoptosis induced by serum deprivation or by ceramide in NIH 3T3 fibroblasts [4], and PC12 cells[6]. In the latter cells, the anti-apoptotic effect correlated with inhibition of caspases-2, -3, and -7 and of the stress-activated protein kinase, JNK [6]. Collectively, these observations implicate SPHK1 and S1P formation in cell growth, transformation, and cancer.

Previously it has been shown that S1P induces proliferation of human breast carcinoma MCF-7 cells [7]. In addition, in MCF-7 cells, overexpression of SPHK1 inhibited chemotactic motility compared with vector-transfected cells and markedly increased cellular S1P levels in the absence of detectable secretion[8]. These results suggest that the inhibitory effect of S1P on chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of S1P rather than through cell surface receptors. It was examined whether SPHK1 contributes to tumorigenesis of breast cancer

MCF-7 cells ex vivo and in nude mice. We found that overexpression of SPHK1 confers a growth advantage to MCF-7 cells by an estrogen-dependent mechanism related to activation of extracellular signal-related kinases (ERK) and furthermore promotes tumorigenesis of these cells in nude mice and neovascularization of the tumors.

BODY

Overexpression of Sphingosine Kinase-1 Promotes Growth and Survival of Human Breast Cancer MCF-7 Cells:

Recently, Dr. Olivera in our lab demonstrated that sphingosine kinase-1 (SPHK-1) promotes cell growth and survival in non-transformed NIH 3T3 fibroblasts[4]. Studying various human breast cancer cell lines did not reveal a correlation between levels of SPHK1 and cell growth or invasive phenotype. MCF-7 human breast cancer (HBC) cells were utilized for subsequent studies as they are readily transfectable and our lab has extensive experience with this cell line. Several MCF-7 clones stably expressing SPHK1 have been generated and I am now examining whether overexpression of SPHK-1 contributes to tumorigenesis of breast cancer MCF-7 cells. Overexpression of SPHK-1 in MCF-7 cells reduced cell death induced by the anti-cancer drug doxorubicin, sphingosine and TNF- α , all known to elevate levels of ceramide, another sphingolipid metabolite implicated in apoptosis. Furthermore, MCF-7 cells overexpressing SPHK-1 are also more resistant to the cytotoxic effects of N,N-dimethylsphingosine, a specific inhibitor of sphingosine kinase. **(Figure 1)** In addition, SPHK-1 confers a small growth advantage to MCF-7 cells as determined by growth in soft agar and by proliferation assays **(Figure 2)**.

Although no changes in estrogen receptor levels could be detected, estrogen antagonists eliminated this growth advantage.

Sphingosine Kinase Overexpression Enhances Survival of MCF-7 Cells

Tumor progression depends on a balance between cell survival and cell proliferation. Apoptosis of non-transformed NIH 3T3 fibroblasts is reduced by overexpression of SPHK1 [5, 9]. However, much less is known of the role of SPHK1 in apoptosis of cancer cells. We previously found that breast adenocarcinoma MCF-7 cells overexpressing murine SPHK1 (MCF-7/SPHK1) have elevated intracellular S1P levels but are unable to release S1P to the extracellular milieu [8]. We have now examined the effects of various stress stimuli on growth and survival of these cells. MCF-7/SPHK1 cells are more resistant than control cells to death induced by the antineoplastic agent doxorubicin, or TNF- α in the presence of cyclohexamide, which sensitizes cells to the toxic effect of TNF- α (**Figure 1**). Previously, we have shown that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin and itself induces apoptosis in MCF-7 cells [10]. In agreement, sphingosine markedly induced apoptosis of MCF-7 cells and there was significantly less apoptosis in sphingosine treated MCF-7/SPHK1 cells (**Fig. 1C**), indicating that increased intracellular S1P levels protect MCF-7 cells against sphingosine-induced apoptosis. Further confirming that the cytoprotective effect was due to the enzymatic activity of SPHK1, N,N-dimethylsphingosine (DMS), a potent competitive SPHK inhibitor[11], reduced the apoptosis sparing effect (**Fig. 1D**). In contrast to NIH 3T3 fibroblasts, in which serum deprivation markedly induced cell death

that was abrogated by enforced SPHK1 expression, MCF-7 cells are not sensitive to serum withdrawal (**Fig. 1E**).

Sphingosine Kinase-1 Overexpression Confers Growth Advantage to MCF-7 Cells

Because the most well known biological effect of S1P is stimulation of proliferation, we next examined the effect of enforced expression of SPHK1 on growth of MCF-7 cells. Overexpression of SPHK1 enhanced proliferation of MCF-7 cells in the presence of 10 % but not in 0.5 % fetal bovine serum (**Fig. 2A**). Analyses of the growth curves during the exponential growth phase revealed that SPHK1 expression decreased the doubling time from 37.7 ± 3.3 h to 28.0 ± 2.9 h. The growth advantage of MCF-7/SPHK1 was obliterated in a dose-dependent manner by DMS, suggesting that the proliferation stimulating effect of SPHK1 depends on its enzymatic activity (**Fig. 2B**).

Previously, it has been shown that NIH 3T3 fibroblasts overexpressing SPHK1 acquire a transformed phenotype as determined by anchorage-independent growth in soft agar assays. Similarly, MCF-7/SPHK1 cells formed more and larger colonies than vector cells when grown in agar in the presence of serum (**Fig. 2C**). However, no colonies were formed when MCF-7/SPHK1 cells were plated in serum-free agar (data not shown), suggesting that factors present in serum are required for SPHK1-induced transformation.

The mitogenic effects of S1P stem from binding to specific cell surface receptors [12, 13] or to intracellular actions [14, 15] depending on the cell type and expression of receptors. Previously, it has been reported that MCF-7 cells proliferate in response to the addition of S1P through activation of EDG-3/S1P₃ and EDG-5/S1P₂ expressed on these cells. However, intracellular levels of S1P were elevated in MCF-7/SPHK1 cells but no measurable release into the media could be detected. DihydroS1P also binds to and

stimulates all of the S1PRs but in contrast to S1P has no known second messenger action and thus is useful to differentiate between receptor-driven effects and intracellular actions. At nanomolar concentration, neither S1P nor dihydroS1P induced significant proliferation of parental MCF-7 cells cultured in 5 % CCS (**Fig. 3A**), whereas overexpression of SPHK1 still enhanced growth in these conditions, albeit not as effectively as in 10% FBS (**Fig. 3B**). Although previously we and others have found that S1P also induced a moderate increase in DNA synthesis of MCF-7 cells as measured by [³H] thymidine incorporation, no significant increase in cell numbers was detected even when MCF-7 cells were cultured for 8 days in 10% FBS containing S1P or dihydroS1P. It was previously suggested that exogenous S1P enhanced growth of MCF-7 cells partly by enhancing secretion of type II insulin-like growth factor (IGF-II), as monoclonal anti-IGF-II and anti-IGFR1 antibodies strongly suppressed proliferation induced by S1P. However, in the presence of serum where overexpression of SPHK1 significantly enhanced cell growth, addition of anti-IGF-IR antibody which blocks the effects of both IGF-I and IGF-II, did not have a marked effect on proliferation during eight days of culture. Collectively, these results suggest that the mitogenic effect of SPHK1 and intracellularly generated S1P might not be due to activation of S1P receptor signaling present in MCF-7.

Molecular Basis for Crosstalk between S1P1/EDG-1 and PDGF activation of SPHK1:

Aberrant FAK Phosphorylation and Activation.

The observation that deletion of *edg-1* does not affect proliferative or survival responses to PDGF, yet eliminates PDGF-mediated motility, suggests that the point of signal disruption lies downstream of the PDGFR. Indeed, there were no significant differences in PDGF-induced tyrosine phosphorylation of PDGFR in EDG-1 ^{-/-} compared

to wild-type fibroblasts, reaching a maximum level within 5 min and decreasing thereafter (**Fig. 4**). Interestingly, in wild type MEFs, PDGF markedly stimulated tyrosine phosphorylation of proteins with molecular weights of 125 and approximately 60 kDa which co-migrated with FAK and Src, respectively.

FAK has been implicated in organization and turnover of focal adhesions and is a receptor-proximal sensor that integrates PDGFR, GPCR, and integrin signals required for cell migration [16]. In agreement with other studies, in wild-type fibroblasts, PDGF rapidly increased phosphorylation of cytoskeleton associated FAK on Y577 (**Fig. 5**), which is located in the kinase catalytic domain and is required for maximal activity; whereas in EDG-1 $-/-$ MEFs, PDGF had no effect on tyrosine phosphorylation of FAK which appeared to be constitutively hyperphosphorylated (**Fig. 5**). In agreement with previous studies demonstrating that FAK functions as part of a large cytoskeleton-associated network of signaling proteins, which includes the Src family tyrosine kinases, p130Cas and paxillin[17], PDGF rapidly induced translocation of p130Cas and paxillin to focal adhesions in wild type MEFs (**Fig. 6**). Notably, similar to FAK $-/-$ cells, levels of the focal adhesion components paxillin and Cas associated with the cytoskeleton appeared to be enhanced in EDG-1 $-/-$ cells and not regulated by PDGF (**Fig. 6**).

***Edg-1* Deletion Abrogates PDGF-Induced Src Activation**

Because active recruitment and activation of Src-family protein tyrosine kinases (Src, Yes, and Fyn, hereafter referred to as Src) to FAK at its phosphorylated Y397 site may be the first of several signaling events necessary to promote PDGF-stimulated cell migration[16, 18, 19], it was important to determine whether the migration defect might also be related to activation of Src. In wild type MEFs, similar to previous reports, PDGF

induced activation of cytoskeleton-associated Src within 5 min (**Fig. 7**), as determined by Western blotting with an antibody specific for phosphotyrosine 418, an autophosphorylation site located in the Src catalytic domain required for full activity. In contrast, basal Src activation was higher and PDGF did not further increase Y418 phosphorylation in the S1P1/EDG-1 deleted cells, even after 60 min.

KEY RESEARCH ACCOMPLISHMENTS

- Overexpression of SPHK-1 in MCF-7 cells reduced cell death induced by anti-cancer drugs, sphingosine, and TNF- α
- Overexpression of SPHK-1 confers a small growth advantage to MCF-7 cells as determined by growth in soft agar and by proliferation assays
- Demonstrated that S1P1/EDG-1 is not required for cell growth or cell survival.
- Accumulated abundant evidence demonstrating that S1P induces migration via S1P1/EDG-1.
- Demonstrated that FAK, Src, and p38 are all necessary downstream elements involved in PDGF activation of SPHK and the subsequent signaling from S1P1/EDG-1.
- Demonstrated that tumors implanted in mice that were overexpressing SPHK had a greater number of peripheral blood vessels compared to vector transfected cells.

REPORTABLE OUTCOMES

PAPERS

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ABSTRACTS

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Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, Milstien S, Spiegel S. Transactivation of the sphingosine-1-phosphate receptor EDG-1 is required for PDGF-induced cell motility. Student Research Days, Georgetown University. 2000 Washington, DC.

Hobson JP, Rosenfeldt HM, Barak LS, Olivera A, Poulton S, Caron MG, Milstien S, Spiegel S. Transactivation of the sphingosine-1-phosphate receptor EDG-1 is required for PDGF-induced cell motility. Society for Experimental Biology, Graduate Student Research Forum. April 6, 2000. Washington, DC.

CONCLUSIONS

Tumor growth is ensured by many oncogenic traits: cell growth independent of growth signals; insensitivity to anti-growth signals; apoptosis protection; angiogenesis; the ability to replicate limitlessly; and invasion/metastasis. S1P, the product of SPHK, may be involved in oncogenesis since it may contribute to three of these traits depending on the cell type involved. First, elevated S1P levels promote cell proliferation in non-transformed cells in the absence of growth factors and it has been suggested that SPHK is an oncogene, acting in a Ras-dependent signaling pathway[5]. Second, elevating S1P levels by either exogenous addition or by SPHK overexpression, acts upstream of caspase activation to inhibit apoptosis induced by a variety of stimuli [4, 6]. This suggests that SPHK, inhibits the apoptotic cascade upstream of the release of these mitochondrial apoptogenic factors [20]. Finally, S1P may promote angiogenesis as suggested by formation of endothelial tubes *ex vivo*[1], by enhancement of mature neovessels *in vivo* [21, 22], and by defective maturation of blood vessels in mice with a deletion of the S1P receptor Edg-1/S1P₁[23]. Therefore, it is important to characterize the role of S1P derived from SPHK in the development of cancer.

Increased resistance to apoptosis is a hallmark of most types of cancer. MCF-7 cells have a deletion of caspase-3 and utilize caspase-7 to execute programmed cell death[10]. These results support a role for SPHK in regulation of cell survival to promote tumor growth. Inhibition of caspase-7 activation and PARP cleavage, correlated with enhanced survival of MCF-7/SPHK1 cells after treatment with doxorubicin, TNF- α , sphingosine or DMS. Our results suggest that SPHK1 enhances the resistance of MCF-7 cells to apoptotic stimuli by curtailing Bax, and reducing JNK activation.

In agreement, the anti-apoptotic effect of S1P has been correlated with downregulation of Bax expression in Jurkat cells[24] and inhibition of phosphorylation of JNK in PC12 cells[6]. It seems most likely that the effects of SPHK1/S1P on survival are mediated through intracellular actions because in many cell types, dihydro-S1P, which binds and activates all of the Edg/S1Pr family, does not protect from apoptosis[6, 25, 26]. However, compelling evidence indicates that SPHK1/S1P is able to promote cell growth not only by intracellular[14, 27] but also by extracellular actions[12, 28, 29]. A clear differentiation of these actions may be confounded by the recent report that S1P activates sphingosine kinase and stimulates its own production after Edg/S1P receptor engagement[30]. Conversely, intracellularly generated S1P is able to stimulate Edg/S1P receptors in an autocrine or paracrine fashion, even when secretion is undetectable by mass level analysis[31]. In contrast, elevated S1P concentrations have been detected in ascitic fluid from ovarian cancer patients [32]. However, several lines of evidence suggest that SPHK1-promoted proliferation of MCF-7 cells is independent of binding of S1P to its receptors. First, neither S1P or dihydroS1P stimulate growth of parental MCF-7 cells. Second, SPHK1/MCF-7 cells do not release detectable amounts of S1P into the medium. Third, previously it has been suggested that proliferation of MCF-7 cells induced by activation of S1PRs is due to enhanced secretion of insulin-like growth factor (IGF-II) and activation of IGF-IR[33], known to induce Akt-dependent mitogenesis, anti-IGF-IR did not block proliferation induced by SPHK1 and its expression did not increase phosphorylation of Akt. However, it still remains possible that spatial and temporal generation of S1P in the vicinity of its receptors may activate ERK leading to proliferation. The proliferative advantage attributable to SPHK1 together with its anti-apoptotic function,

may favor tumor progression. Indeed, MCF-7 cells overexpressing SPHK1 exhibit robust anchorage-independent growth and result in improved tumorigenesis in nude mice.

Estrogens strongly promote proliferation of mammary cells after binding to ER. Malignant progression of breast cancer correlates with acquisition of estrogen-independent growth by mechanisms that are not fully understood [34]. It is thus important to understand signaling cooperation in estrogen-dependent breast cancer cells, such as MCF-7 cells, in order to identify cross-talk pathways that could impinge on cancer progression. Interestingly, SPHK1 enhances the growth effects of estradiol, and ICI inhibits the mitogenic effects of SPHK1. However, SPHK1 does not affect ER levels or regulation of estrogen responsive genes, and conversely, estradiol has no effect on SPHK1 activity, suggesting that S1P may act independently. Taken together, these results suggest a mechanism whereby S1P promotes estrogen-dependent tumor progression. Interestingly, constitutively active Ras mutations, which stimulate ERK, also activate SPHK1[5], raising the possibility of a positive feedback loop involving ERK that ensures optimal cell growth. It is noteworthy, that SPHK1 stimulates the growth of MCF-7 cells cultured in full serum. This suggests that in addition to a role initiating oncogenic transformation, S1P could also promote the growth of established tumors.

Angiogenesis, the formation of new blood vessels, is an essential requirement for tumor growth. S1P promotes vessel formation through Edg-1 mediated mechanisms that are sensitive to pertussis toxin and C3 exoenzyme[1, 21, 22, 35]. Disruption of the Edg-1/S1P₁ gene in the mouse results in embryonic lethality due to a vascular maturation defect. Moreover, it has been suggested that synergism between Edg-1/S1P₁, Edg-3/S1P₃ and various angiogenic factors such as vascular endothelial cell growth factor (VEGF) and

fibroblast growth factor (FGF) is necessary for mature neovessel formation in vivo in the presence of S1P[21]. In agreement we detected an increase in edge-associated microvessels in MCF-7 tumors overexpressing SPHK1. Dramatically enhanced neovascularization by SPHK1 adenovirus or SPHK1 transduced AE1-2a cells implanted into the Matrigel plugs containing FGF-2 has been reported. In contrast, the effect of SPHK1 overexpression on new blood vessel formation in MCF-7 derived tumors was much smaller. This could be due to differences in cell type although in both cases, there was no detectable secretion of S1P. Thus, although we cannot exclude a paracrine contribution of S1P on endothelial cells invading the tumors, this is likely not the major factor contributing to increased numbers and larger tumors produced by MCF-7/SPHK1 cells. Collectively, our results suggest that SPHK1 may play an important role in breast cancer progression by regulating tumor cell growth and survival. If so, SPHK inhibition might be a useful adjunct to anti-estrogen treatment of breast cancer.

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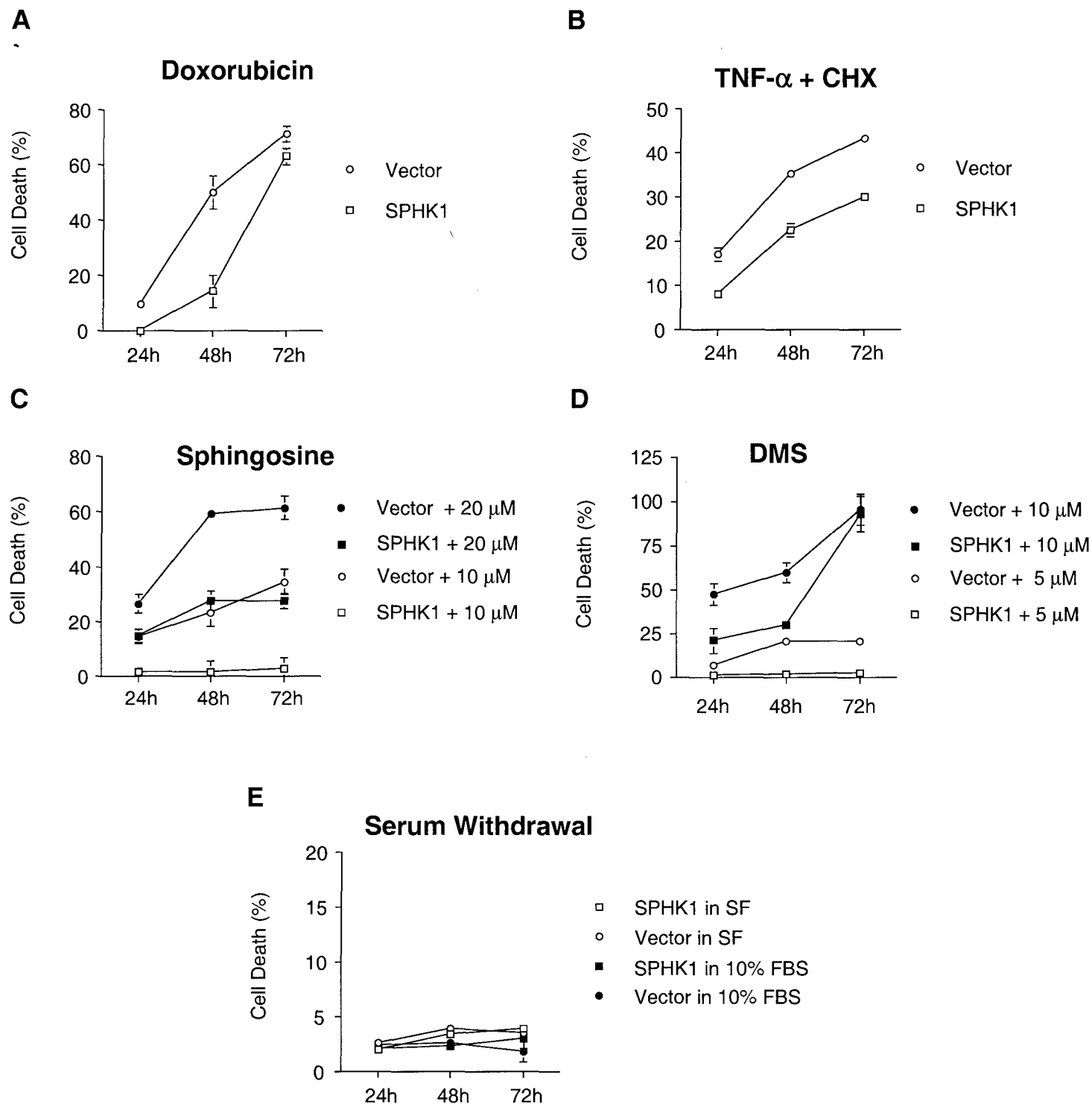


Figure 1

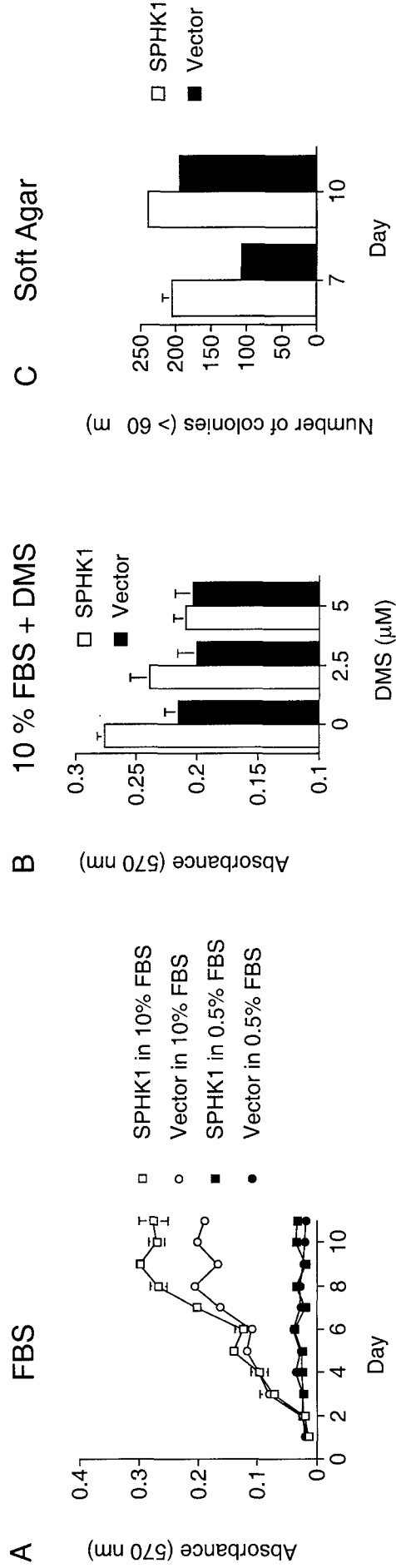


Figure 2

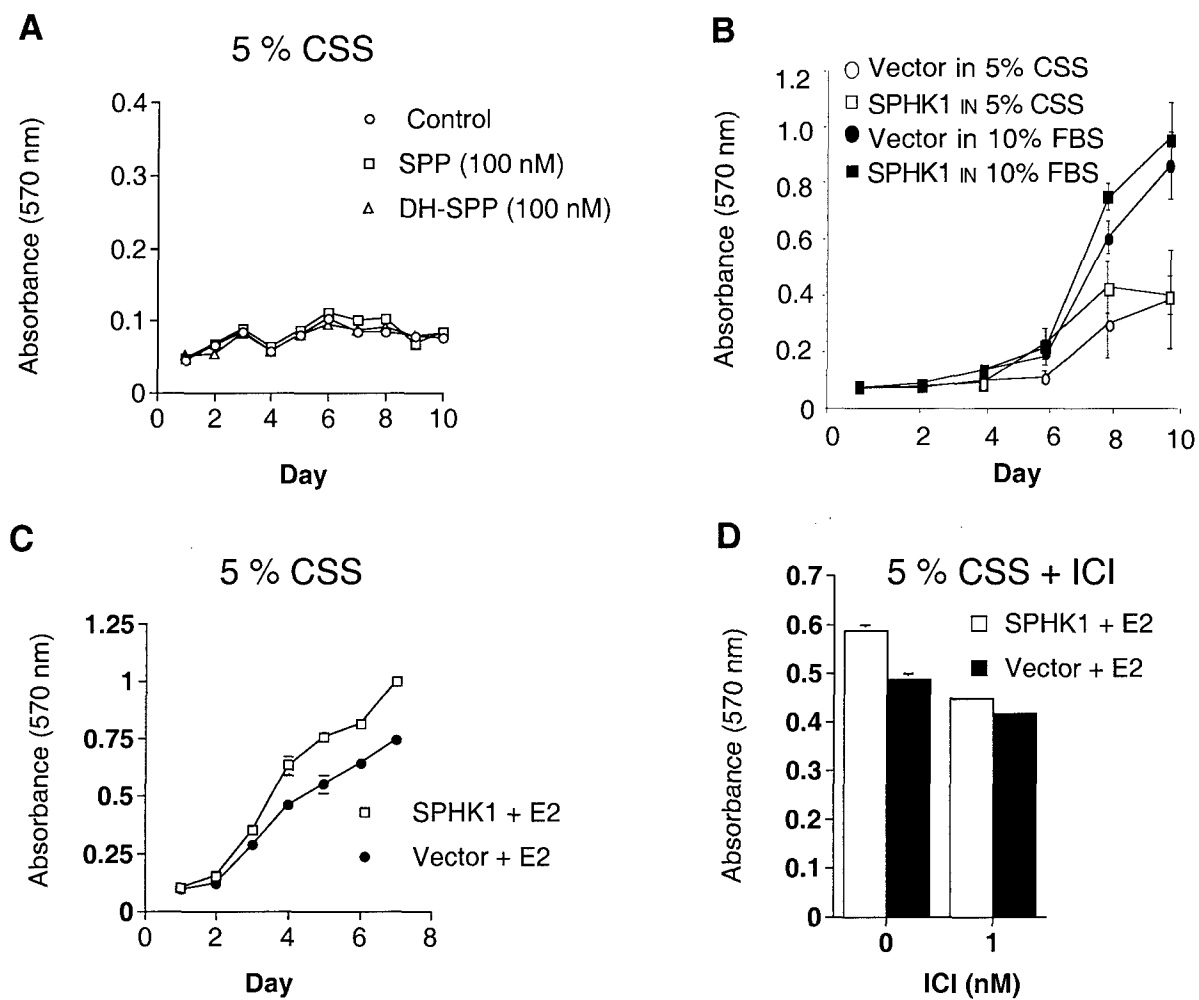


Figure 3

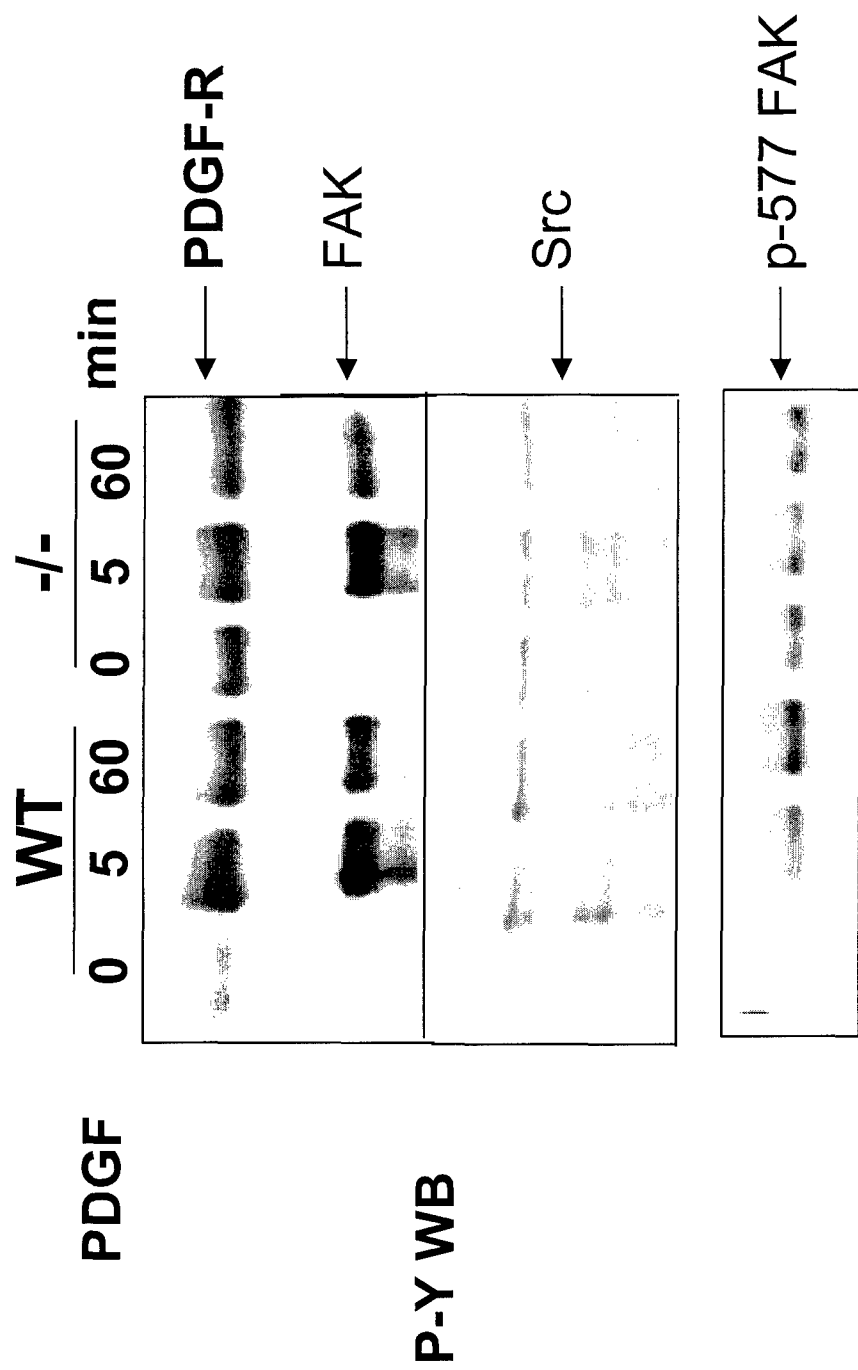


Figure 4

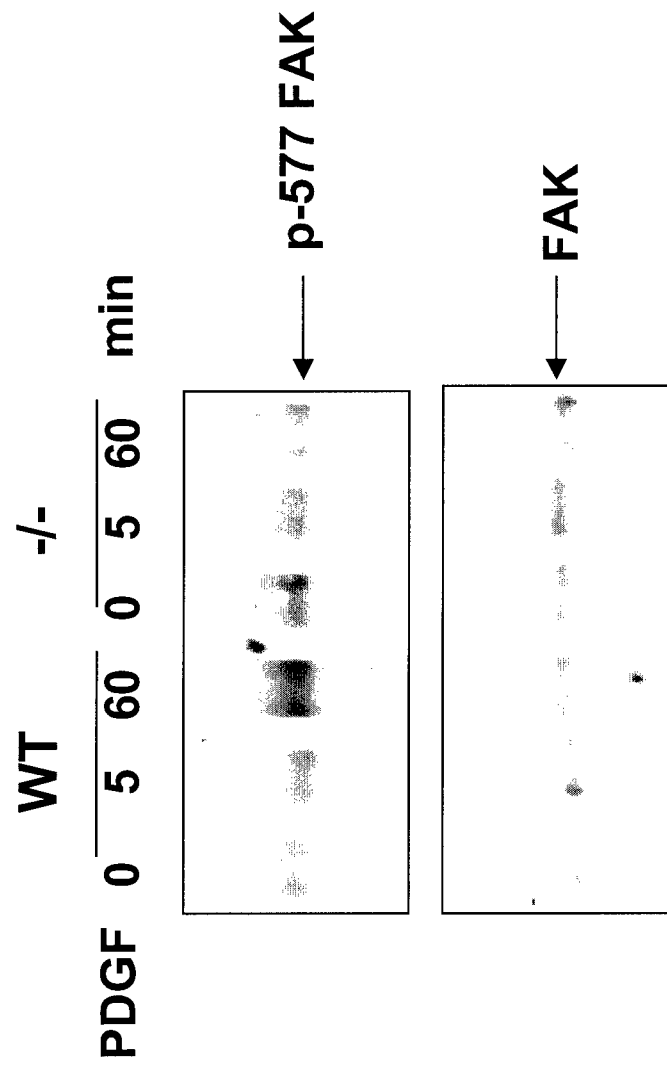


Figure 5



Figure 6

A

B



Figure 7

**EDG-1 LINKS THE PDGF RECEPTOR TO SRC AND FOCAL ADHESION KINASE
ACTIVATION LEADING TO LAMELLIPODIA FORMATION AND CELL MIGRATION**

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Running Title: EDG-1 in PDGF-R signalingp

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ABSTRACT

Sphingosine-1-phosphate (SPP), formed by sphingosine kinase, is the ligand for EDG-1, a GPCR important for cell migration and vascular maturation. Here we show that cytoskeletal rearrangements, lamellipodia extensions and cell motility induced by PDGF are abrogated in EDG-1 null fibroblasts. However, EDG-1 appears to be dispensable for mitogenicity and survival effects, even those induced by its ligand SPP and PDGF. Furthermore, PDGF induced focal adhesion formation and activation of FAK, Src, and stress activated protein kinase-2, p38, were disregulated in the absence of EDG-1. In contrast, tyrosine phosphorylation of the PDGFR and activation of extracellular signal regulated kinase (ERK1/2), important for growth and survival, were unaltered. Our results suggest that EDG-1 functions as an integrator linking the PDGFR to lamellipodia extension and cell migration. PDGF, which stimulates sphingosine kinase, leading to increased SPP levels in many cell types, also induces translocation of sphingosine kinase to membrane ruffles. Hence, recruitment of sphingosine kinase to the cell's leading edge and localized formation of SPP may spatially and temporally stimulate EDG-1 resulting in activation and integration of downstream signals important for directional movement toward chemoattractants, such as PDGF. These results may also shed light on the vital role of EDG-1 in vascular maturation.

KEY WORDS: EDG-1, sphingosine-1-phosphate, motility, Src, FAK, PDGF

INTRODUCTION

The bioactive sphingolipid metabolite sphingosine-1-phosphate (SPP), formed by activation of sphingosine kinase in response to diverse stimuli, is the ligand for the Endothelial Differentiation Gene-1 (EDG-1) family of GPCRs (reviewed in (1-3)). These receptors, which include EDG-1, -3, -5, -6 and -8, all bind SPP and dihydroSPP with high affinity but couple to different G-proteins and thus regulate diverse processes. Whereas EDG-1, EDG-6 and EDG-8 couple mainly to G_i , both EDG-3 and EDG-5 activate G_i , G_q , and $G_{12/13}$ (reviewed in (1-4)). Although these receptors are differentially expressed, surprisingly they all have been implicated in cell migration (5-9). Activation of EDG-1 or EDG-3 by SPP or dihydroSPP in many cell types induces chemotaxis, whereas binding of SPP to EDG-5 abolished directed chemotaxis and membrane ruffling (5,10-13). Members of the EDG-1 family differentially regulate the small GTPases of the Rho family, particularly Rho and Rac (14), which are downstream of the heterotrimeric G-proteins and are important for cytoskeletal rearrangements (15,16). Binding of SPP to EDG-1 regulates Rac-coupled cortical actin formation (14), while binding to EDG-3 and EDG-5 elicits Rho-coupled stress fiber assembly (10), and EDG-5 also negatively regulates Rac activity (10), thereby inhibiting cell migration.

Cell movement is essential throughout life, particularly during development, and is also important in many physiological and pathological processes, including inflammation, wound healing, tumor growth, metastasis, and angiogenesis. A mutation in the zebrafish homologue of the *edg-5* gene, miles apart, was shown to cause defective migration of myocardial cells during vertebrate heart development, revealing a unique role for EDG-5 in regulating cell migration in organogenesis of the heart (9).

Disruption of the *edg-1* gene in mice by Proia and colleagues revealed that SPP/EDG-1 signaling is essential for vascular maturation (17). Remarkably, although EDG-1 null embryos died *in utero* due to massive hemorrhage, they had normal vasculogenesis and a substantially normal blood vessel network, yet they were severely impaired in recruitment of smooth muscle cells and pericytes to the vessel walls presumably due to their defective migration (17). Recently, we found that migration of cells from these embryos toward SPP (17) and platelet-derived growth factor (PDGF) (18), which stimulates sphingosine kinase and increases intracellular SPP in many cell types (19), was dependent on expression of EDG-1. Moreover, PDGF activated EDG-1, as measured by its phosphorylation and translocation of β -arrestin, suggesting a new mechanistic concept for cross communication between a tyrosine kinase receptor, PDGFR, and a GPCR such as EDG-1 (18). In this study, we utilized EDG-1 null fibroblasts to determine the role of EDG-1 in cell growth and survival mediated by SPP and PDGF and examined the molecular mechanisms whereby EDG-1 so dramatically influences directed cell movement.

METHODS

Cell culture

MEFs were derived from wild type and EDG-1 $-/-$ day 8.5 embryos (17).

Embryos were dissected, rinsed with phosphate-buffered saline (PBS), minced, and digested with trypsin (2 min at 37 °C in 2.5% trypsin/1 mM EDTA). Trypsin was inactivated by addition of DMEM containing 10% FBS. Cells were subcultured every 3 days for a maximum of ten passages. Only MEFs from passages 2-9 were used for experiments. Immortalized fibroblasts were obtained from spontaneously transformed MEFs after continuous culture.

DNA synthesis

[^3H]Thymidine incorporation into DNA was measured as described (20). Values are the means of triplicate determinations and standard deviations were routinely less than 10% of the mean.

Determination of apoptotic cells

Wild type and EDG-1 $-/-$ embryonic fibroblasts were plated in 24 well clusters at a density of 1×10^4 cells/well in DMEM containing 10% FBS, washed and incubated in serum-free media containing the indicated agents for 48 h. Cells were then fixed, stained with Hoechst and apoptosis assessed as previously described (20). In some experiments, viable cells were determined by trypan blue exclusion.

Chemotactic motility

Chemotaxis was measured in a modified Boyden chamber as previously described using polycarbonate filters (25 X 80 mm, 12 μm pore size) coated with collagen type I (50 $\mu\text{g/ml}$ in 5% acetic acid) (5). Chemoattractants were added to the

lower chamber and cells added to the upper chamber at 5×10^4 cells per well. After 18 h, non-migratory cells on the upper membrane surface were mechanically removed and the cells that traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik. The number of migratory cells per membrane was counted using a microscope with a 20X objective. Each data point is the average number of cells in four random fields, each counted twice and is the average \pm S.D. of three individual wells.

In vitro wound healing assay

Confluent fibroblast monolayers were wounded by scraping with a pipette tip, washed twice to remove detached cells, and then incubated in serum-free DMEM containing 0.1% BSA. After 12 h, cells were fixed with 2% glutaraldehyde in PBS and photographed.

Western blotting

Fibroblasts were scraped in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin). In some experiments, the cytoskeleton associated Triton X-100-insoluble fraction was recovered by centrifugation (14,000 \times g, 10 min, 4 °C) and resuspended in lysis buffer supplemented with 1% SDS. Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose. Anti-paxillin, anti-Cas and FAK (Transduction Labs), c-Src GD11 antibody (Upstate Biotechnology, Lake Placid, NY), and anti-Src [pY⁴¹⁸] and FAK [pY⁵⁷⁷] antibodies (Biosource, Rockville, MD), phospho-p38, p38, and phospho-ERK1/2 antibodies (New England Biolabs), anti-PDGF receptor

polyclonal antibody and phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) were used as primary antibodies. Immunocomplexes were visualized by enhanced chemiluminescence (ECL) as described (20).

Immunostaining

Cells grown on glass coverslips coated with 50 µg/ml collagen I were incubated overnight in DMEM. Cells were fixed in 3.7% formaldehyde for 30 min at room temperature, and permeabilized in 0.5% Triton-X100 for 5 min. Actin filaments were visualized with Alexa 488-conjugated phalloidin (Molecular Probes, Eugene, OR) and focal complexes with antibodies to vinculin (Upstate Biotechnology, Lake Placid, NY), followed by staining with rhodamine-conjugated secondary antibody. After washing three times with PBS, coverslips were mounted on slides using an Anti-Fade kit (Molecular Probes) and cells were examined by confocal microscopy. Where indicated, cells were transfected with 5 µg of GFP-SPHK1 fusion plasmid as described (20), treated with PDGF for 5 min, fixed and visualized by confocal fluorescence microscopy.

Src Kinase Assay

Cells were lysed in RIPA buffer (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM HEPES (pH 7.4), 4 mM EGTA, 2 mM EDTA, 2 mM NaVO₄, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 1 mM PMSF) and insoluble material was pelleted. Supernatants were precleared by incubation with a 1:1 Protein A/G (Santa Cruz) slurry for 1 h at 4° C, followed by brief centrifugation to remove the beads. Either Src or Yes monoclonal antibodies or Fyn polyclonal antibody was added to lysates and samples incubated for 1 h at 4° C. Antibody complexes were precipitated with Protein A/G. Beads were washed in RIPA and then in buffer containing 50 mM PIPES-KOH (pH 7.0), 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0.5 mM PMSF. Kinase reactions were carried out using a synthetic

peptide corresponding to amino acids 6-20 of p34^{cdc2}. Reaction solution (25 μ l) containing 500 μ M peptide, 200 μ M ATP, and 0.4 mCi/ml γ -³²P-ATP was added to 25 μ l of each sample. In each case, the activity in the absence of substrate peptide was also determined. After 10 min incubations at room temperature, reactions were stopped by centrifugation and spotting an aliquot of the supernatants on Whatman P81 filter paper. Filters were washed in 1% phosphoric acid and radioactivity determined by liquid scintillation counting.

RESULTS

Cell Migration is Defective in EDG-1 Null Fibroblasts

Recently, we have shown that cell migration toward PDGF, a growth factor which stimulates sphingosine kinase and increases SPP, was dependent on expression of EDG-1. Conversely, deletion of EDG-1 markedly reduced chemotaxis toward PDGF (18). Thus, it was of interest to examine whether this was a PDGF-specific effect or whether migration toward other growth factors and chemoattractants was also EDG-1-dependent. Fibroblasts isolated from EDG-1 knockout mouse embryos (17), not only did not migrate toward PDGF, but also had reduced chemotaxis to IGF-1, and thrombin, while migration toward EDG was also reduced, albeit to much lesser extent (Fig. 1A). However, migration toward fibronectin was unaffected, suggesting that, in contrast to chemotaxis-driven motility, EDG-1 is not required for haptotactic cell migration.

In the Boyden chamber cell migration assay, differences in cell shape and size may affect passage through the pores in the membrane. These properties do not affect the wound healing assay which can also be used to qualitatively compare cell migration. Thus, after making a wound in a monolayer of cells, wild-type mouse embryonic fibroblasts (MEFs) rapidly migrated into the wounded area, whereas EDG-1 $-/-$ fibroblasts were not able to actively invade the wound (Fig. 1B). To further examine the role of EDG-1 in cell migration, we established immortalized fibroblast lines from wild-type and EDG-1 $-/-$ MEFs. The immortalized EDG-1 $-/-$ cell line retained the severe migratory defects previously noted in the MEFs ((18) and Fig. 1), not only toward SPP and PDGF, but also toward IGF-1 (Fig. 1C). Similar to our previous results with wild-type MEFs (18), treatment with pertussis toxin to inactivate G_i -coupled EDG-1 also

markedly reduced migration of these EDG-1 +/+ immortalized cells toward SPP and PDGF (data not shown). Furthermore, the migratory defect is clearly related to the lack of expression of EDG-1 as enforced expression of EDG-1 was able to reverse the migratory defect of these cells (Fig. 1D).

SPP- and PDGF-Induced Cellular Proliferation are Independent of EDG-1

Expression

Recent studies suggest that EDG-1 plays a critical role in SPP-stimulated proliferation of endothelial cells (5,14,21), whereas in other cell types, its effect appears to be mediated by intracellular actions (22). We utilized fibroblasts isolated from EDG-1 knockout mouse embryos to definitively determine whether EDG-1 is essential for the mitogenic effects of SPP and PDGF. Unexpectedly, the mitogenic effect of SPP was not abrogated in EDG-1 -/- fibroblasts (Fig. 2A). Moreover, sphingosine, which is readily taken up by cells and converted intracellularly to SPP, was equally as effective in stimulating proliferation of EDG-1 -/- fibroblasts as wild-type fibroblasts. Similarly, the mitogenic effects of PDGF-BB and fetal bovine serum (FBS) were not significantly different in wild-type and EDG-1 -/- MEFs (Fig. 2A). In agreement, no significant differences in DNA synthesis in response to SPP, PDGF, or serum could be detected between wild-type and EDG-1 -/- immortalized fibroblast cell lines (Fig. 2B). These results suggest that EDG-1 function is dispensable for the mitogenic effects of SPP and PDGF.

Mitogen activated protein (MAP) kinase family, ERK, SAPK/JNK and p38 play an important role in cell growth, survival, and motility (23). While it is well established that

ERK activation is required for PDGF-stimulated DNA synthesis, activation of p38 is required for PDGF-induced cell motility and actin reorganization (24). In agreement with previous studies (24), in wild type fibroblasts, PDGF induced sustained activation of ERK1 and ERK2 (Fig. 2C) and a more transient activation of p38 (Fig. 2C), while SAPK1/JNK was not stimulated at all (data not shown). Although EDG-1 deletion had no significant effect on activation of ERK induced by PDGF, it almost completely eliminated p38 activation (Fig. 2C).

Survival effects of SPP and PDGF are not compromised by *EDG-1* deletion

Activation of EDG-1 by SPP has also been shown to protect endothelial cells from apoptosis (14,21) and this survival effect was markedly attenuated by EDG-1, but not EDG-3 or EDG-5, antisense oligonucleotides (14), whereas other studies suggested that suppression of apoptosis was mediated via intracellular actions (20,25-29). Therefore, it was important to examine the cytoprotective effect of SPP in EDG-1 knockout fibroblasts. In agreement with previous studies (20), serum deprivation induced apoptosis in a time-dependent manner, where shrinkage and condensation of nuclei were clearly evident after 48 h (Fig. 3A, B). Disruption of the *edg-1* gene had no significant effect on apoptosis (Fig. 3). Moreover, similar to other cell types (25,26), addition of micromolar concentrations of SPP (Fig. 3A, B), but not nanomolar concentrations (data not shown), to control or EDG-1 *-/-* fibroblasts markedly suppressed apoptosis induced by serum deprivation or the chemotherapeutic drug doxorubicin. These protective effects were specific and unrelated to EDG-1, because dihydro-SPP, which lacks the trans double bond present in SPP yet binds and activates EDG-1 equally well (5,22), did not significantly prevent apoptosis in wild type or mutant

fibroblasts. Moreover, there were also no significant differences in either the extent of the cell death responses or the protection by PDGF or SPP of wild-type and EDG-1 $-/-$ immortalized fibroblast cell lines to serum starvation or other apoptotic stimuli (Fig. 3C), including doxorubicin or TNF- α in the presence of actinomycin D, which sensitizes cells to the toxic effect of TNF- α (30).

EDG-1 Null Fibroblasts Display Aberrant Cytoskeletal Architecture and Focal Contacts In Response to PDGF

Cell movement is a complex process orchestrated by the interplay of leading edge formation and the turnover of the focal adhesions that tether the cell to the extracellular matrix. Leading edge formation is under the control of members of the Rho family of small GTPases (Rac, Cdc42, and Rho) (15,16) and involves actin polymerization and the formation of nascent focal adhesion complexes. The turnover of focal adhesions is modulated by tyrosine kinases that reside within these complexes, such as focal adhesion kinase (FAK) (31) and Src (32-34). To better understand the migratory defect of EDG-1 null fibroblasts toward PDGF, we first examined the architecture of the cytoskeleton and focal adhesion formation. No obvious differences between quiescent wild type and EDG-1 $-/-$ MEFs were revealed by phalloidin staining of actin filaments or by antibodies to the cytoskeleton protein vinculin, a major component of focal adhesions (Fig. 4). However, although PDGF, as expected (15,16,35), caused extension of lamellipodia at the cell periphery of wild type fibroblasts, lamellipodia were almost completely absent in PDGF-treated EDG-1 null fibroblasts (Fig. 4). Moreover, vinculin-positive patches were scattered across the ventral surface of EDG-1 $-/-$ cells, in contrast to the typical focal adhesions at the cell periphery

observed in wild-type cells. Reminiscent of the morphological changes in FAK deleted fibroblasts (31,36), actin fibers were much more dense around the periphery of EDG-1-deficient cells (Fig. 4G), rather than organized in long parallel projections equally distributed throughout the cell as in the wild-type fibroblasts.

Similarly, PDGF did not induce membrane ruffles in immortalized EDG-1 null fibroblasts but instead, triggered the formation of filopodia or microspikes (Fig. 5A,B). To further substantiate the role of EDG-1 in PDGF-induced lamellipodia extension, immortalized EDG-1 null fibroblasts were transfected with GFP-EDG-1 and treated with PDGF (Fig. 5). In contrast to untransfected cells, GFP-EDG-1 transfected EDG-1 null fibroblasts exhibited copious ruffling upon PDGF treatment (Fig. 5D,F).

PDGF Induces Translocation of Sphingosine Kinase to Ruffles

The acquisition of spatial and functional asymmetry between the front and the rear of the cell is a necessary step for directional migration. It has been suggested that components of G protein receptor systems may accumulate at the front of polarized cells accounting for increased responsiveness to chemoattractants at the anterior (37-39). Nonetheless, chemoattractant receptors remain evenly distributed on the surface of polarized cells (37,39) and intermediate intracellular signals that are important for directional migration may be produced in a spatial and temporal manner. Thus, it was tempting to speculate that PDGF might elicit this steep signaling gradient by recruitment of sphingosine kinase to the leading edge where local formation of SPP could result in restricted activation of EDG-1. Indeed, although sphingosine kinase is diffusely distributed in the cytosol of unstimulated cells, PDGF rapidly induced translocation to membrane ruffles as visualized with a SPHK-GFP fusion protein (Fig. 6). Of note,

sphingosine, the substrate of SPHK, is a membrane-bound lipid. Hence, recruitment of SPHK to membrane ruffles should generate SPP in a spatially restricted manner.

Molecular Basis for Crosstalk between EDG-1 and PDGFR Signaling: Aberrant FAK Phosphorylation and Activation.

The observation that deletion of *edg-1* does not affect proliferative or survival responses to PDGF, yet eliminates PDGF-mediated motility, suggests that the point of signal disruption lies downstream of the PDGFR. Indeed, there were no significant differences in PDGF-induced tyrosine phosphorylation of PDGFR in EDG-1 ^{-/-} compared to wild-type fibroblasts, reaching a maximum level within 5 min and decreasing thereafter (Fig. 7A). Interestingly, in wild type MEFs, PDGF markedly stimulated tyrosine phosphorylation of proteins with molecular weights of 125 and approximately 60 kDa which co-migrated with FAK and Src, respectively.

FAK has been implicated in organization and turnover of focal adhesions and is a receptor-proximal sensor that integrates PDGFR, GPCR, and integrin signals required for cell migration (34,36,40). In agreement with other studies (34), in wild-type fibroblasts, PDGF rapidly increased phosphorylation of cytoskeleton associated FAK on Y577 (Fig. 7B), which is located in the kinase catalytic domain and is required for maximal activity; whereas in EDG-1 ^{-/-} MEFs, PDGF had no effect on tyrosine phosphorylation of FAK which appeared to be constitutively hyperphosphorylated (Fig. 7B). In agreement with previous studies demonstrating that FAK functions as part of a large cytoskeleton-associated network of signaling proteins, which includes the Src family tyrosine kinases, p130Cas and paxillin (41), PDGF rapidly induced translocation of p130Cas and paxillin to focal adhesions in wild type MEFs (Fig. 7C). Notably, similar

to FAK $-/-$ cells, levels of the focal adhesion components paxillin and Cas associated with the cytoskeleton appeared to be enhanced in EDG-1 $-/-$ cells and not regulated by PDGF (Fig. 7C).

***Edg-1* Deletion Abrogates PDGF-Induced Src Activation**

Because active recruitment and activation of Src-family protein tyrosine kinases (Src, Yes, and Fyn, hereafter referred to as Src) to FAK at its phosphorylated Y397 site may be the first of several signaling events necessary to promote PDGF-stimulated cell migration (32,36,42,43), it was important to determine whether the migration defect might also be related to activation of Src. In wild type MEFs, similar to previous reports (reviewed in (34)), PDGF induced activation of cytoskeleton-associated Src within 5 min (Fig. 8A), as determined by Western blotting with an antibody specific for phosphotyrosine 418, an autophosphorylation site located in the Src catalytic domain required for full activity. In contrast, basal Src activation was higher and PDGF did not further increase Y418 phosphorylation in the EDG-1 deleted cells, even after 60 min. Identical results were obtained with immortalized fibroblasts (Fig. 8B). In agreement with previous suggestions that a pertussis toxin sensitive G_i protein regulates activation of Src by PDGF in airway smooth muscle cells (44,45), we found that pertussis toxin inhibited PDGF-induced activation of Src, Fyn, and Yes in wild type cells (Fig. 8C). Although disruption of EDG-1 abrogated the ability of PDGF to stimulate Src, it only had a small effect on Fyn activation (Fig. 8C). Taken together, these results suggest that activation of FAK and Src by PDGF is aberrant in the absence of EDG-1.

DISCUSSION

Role of EDG-1 in Migration but Not in DNA Synthesis or Survival

In this study, we have demonstrated that although the GPCR, EDG-1, plays a critical role in PDGF-induced cell motility, it appears to be dispensable for mitogenicity and survival effects, even those induced by its ligand SPP. Thus, migration of EDG-1 null fibroblasts toward PDGF is markedly inhibited, whereas EDG-1 deletion has no effect on PDGFR functions important for cell cycle progression, including tyrosine phosphorylation of the receptor itself and ERK1/2 activation. In addition, dihydroSPP, which binds and activates EDG-1, did not significantly prevent apoptosis in wild type fibroblasts expressing EDG-1, yet it had similar chemoattracting effects as SPP. Thus, EDG-1 functions in an unprecedented manner as an integrator linking the PDGFR to the cellular machinery of cell migration. Recently, we showed that PDGF transactivated EDG-1 and proposed a new mechanistic concept for cross communication between a tyrosine kinase receptor, PDGFR, and a GPCR such as EDG-1 (18). Further support for this notion recently emerged from the demonstration that PDGFR is tethered to EDG-1 providing a platform for integrative signaling by these receptors (46). According to this paradigm, stimulation of PDGFR by PDGF activates sphingosine kinase resulting in increased formation of SPP, which in turn, stimulates EDG-1 leading to activation of downstream signals critical for cell locomotion (see below). This type of cross-communication seems to be important for microvasculature maturation during development (17), when PDGFR- β positive pericytes are recruited to capillaries from progenitors in the vessel walls through the action of PDGF-BB secreted from endothelial cells (47,48). EDG-1-, PDGF-BB-, and PDGFR- β -deficient embryos all lack

microvascular pericytes/smooth muscle cells surrounding their vessel walls causing their rupture and massive hemorrhaging (17,47,48). Dysfunctional migration of EDG-1 -/- cells toward PDGF links these phenotypes at the final steps of vasculogenesis underscoring the importance of endothelial cell-pericyte communication in vascular maturation. Moreover, our findings that EDG-1 plays a critical role in directed cellular motility may provide the underlying mechanism of the newly discovered role of SPP in *in vivo* angiogenesis (7, 12, 14). Whether other growth factors, such as EGF and VEGF use similar mechanisms to transactivate EDG-1 remains to be determined.

How Does EDG-1 Signaling So Profoundly Affect Directed Cell Movement?

EDG-1 plays a critical role in integrating the responses of several key elements of directed cell movement, including Rac (18), important for lamellipodia formation at the leading edge (15,16,35); FAK and Src, which are necessary for formation and turnover of focal complexes (31-33,36); and stress-activated protein kinase-2 (p38), which is involved in actin reorganization and PDGF-induced cell migration (24).

Although the mechanism whereby the Gi-linked receptor EDG-1 regulates Rac activation in response to PDGF is not well understood, both tyrosine kinases of the Src family and PI3K link G $\beta\gamma$ complexes to activation of Rac via regulation of guanine nucleotide exchange factors (GEFs)(reviewed in (49)). One of these, T-lymphoma invasion and metastasis gene 1 (Tiam1), a specific GEF for Rac (50), might be involved as it is activated by both PDGF and SPP/EDG-1 signaling (14,51). Alternatively, Src, when activated by G $\beta\gamma$ or by recruitment to the membrane mediated by β -arrestin (52,53), or by binding of G α_i to its catalytic domain (54), can directly phosphorylate Ras-

GRF1, thereby inducing GEF activity toward Rac (55). Another candidate for a role in Rac activation is the Cas/Crk complex formed downstream of Src and/or FAK activation (56), as we previously showed that inhibitors of sphingosine kinase suppressed PDGF-induced Crk phosphorylation but did not affect PDGFR autophosphorylation or phosphorylation of the adaptor protein Shc (57). One downstream target of Rac is p38 (58). Indeed, binding of SPP to EDG-1 in several cell types has been shown to activate p38 (7) and inhibitors of this MAP kinase, but not MEK1, the kinase directly upstream of ERK1/2, inhibit motility responses induced by SPP/EDG-1 (59). Furthermore, migration of fibroblasts toward PDGF is inhibited by expression of dominant-negative Rac, while blocking the ERK pathway by dominant-negative MEK1, did not inhibit migration toward PDGF (60). By contrast, migration toward fibronectin was dependent on the ERK pathway but not on Rac, concordant with our finding that EDG-1 deletion also did not markedly affect migration toward fibronectin, indicating that EDG-1 is not important for haptotaxis. Similar to our results, MEFs from mice with a disruption of the gene encoding $G\alpha_{13}$, which resulted in vascular system defects, also showed greatly impaired migratory responses to thrombin, but not to fibronectin (61).

Activation of EDG-1 by SPP stimulates tyrosine phosphorylation of FAK (62) and chemotaxis (5,62). Recent evidence indicates that PDGF promotes phosphorylation of FAK at Y397 creating an SH2-binding site which acts as a "switchable adaptor" to recruit Src to focal adhesion complexes (36). FAK phosphorylation at this indispensable Src binding site functions as part of the cytoskeleton-associated network of signaling molecules downstream not only of PDGFR, but also of integrin and GPCRs, to regulate

cell motility(34,36). Significantly, we previously showed that autophosphorylation of FAK on Y397 is essential for regulation of cell motility by SPP (63).

Migratory deficits were noted in cells lacking Src (64) or FAK and reintroduction of FAK, but not unphosphorylatable mutant Y397F FAK, in FAK deficient cells restored their ability to migrate (36). Because the tyrosine kinase activity of Src has been shown to promote turnover of focal contacts(33), the aberrant cell migration reflects defects in focal adhesion turnover. Notably, PDGF-induced focal adhesion complexes, tyrosine phosphorylation, as well as activation of cytoskeleton-associated Src and FAK, were all disregulated in the absence of EDG-1. This indicates that recruitment and activation of Src by PDGF is dependent on activation of EDG-1. These data provide an explanation for the observation that pertussis toxin inhibits activation of Src by PDGF (44,45). However, it is unlikely that Src is solely responsible for the migratory defects, as recently It was shown that triple null mutations of Src, Yes and Fyn (64), in contrast to the effect of FAK $-/-$ (31,36), had little effect on PDGF-induced cell migration.

G-Protein Coupled Receptor Signaling at the Leading Edge

An early event that marks directional responses of cells is the restricted translocation of the pleckstrin homology (PH) domain containing proteins (indicative of local generation of PIP_3) in a manner similar to the polarity of distribution of $\text{G}\beta\gamma$ subunits along the leading edge (37,38). However, the asymmetric redistribution of $\beta\gamma$ subunits is not sufficiently localized to restrict events to the leading edge (38) and it has been suggested that chemoattractant-associated PH recruitment requires an intermediate pathway dependent on the activity of one or more of the small GTPases

(39). The data presented here identifies a new mechanism that impinges on the signaling cascade that brings about this steep signaling gradient. A tantalizing notion is that recruitment of sphingosine kinase to membrane ruffles and local generation of SPP may convert tyrosine kinase receptor signaling into directed migration. Hence, spatially and temporally restricted generation of SPP in response to PDGF results in restricted activation of the GPCR EDG-1 that, in turn, recruits and activates tyrosine kinases, such as Src and FAK, and the small GTPase Rac at the inner plasma membrane facing the stimulus. This may lead to amplification of signaling at the leading edge of the cell (39).

How do cells generate a steep gradient of SPP?

It is reasonable to assume that synthesis and degradation of SPP are differentially regulated. Enhanced formation of SPP by PDGF could be governed by local recruitment and activation of sphingosine kinase at the ruffles, while global, rapid, and efficient degradation is catalyzed by several types of lipid phosphate phosphatases. The net result would be an asymmetric buildup of SPP at the site of its formation and localized EDG-1/SPP signaling could play a role in directional responses to chemoattractants.

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ABBREVIATIONS

BSA, bovine serum albumin; DMS, N,N-dimethylsphingosine; GFP, green fluorescent protein; PDGF, platelet-derived growth factor; SPP, sphingosine-1-phosphate; EGF, epidermal growth factor; PTX, pertussis toxin; DMEM, Dulbecco's modified Eagle's medium

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FIGURE LEGENDS

Figure 1. *Edg-1* disruption causes aberrant chemotaxis toward PDGF, IGF-1, and thrombin. (A) Wild type (open bars) and EDG-1 $-/-$ MEFs (filled bars) were allowed to migrate toward PDGF-BB (20 ng/ml), EGF (20 ng/ml), IGF-1 (40 ng/ml), thrombin (1 U/ml), or fibronectin (10 μ g/ml) and chemotaxis was measured. Data are means \pm S.D. of triplicate determinations. Similar results were obtained in three independent experiments. (B) **Migration in a wound healing assay.** Wild type and EDG-1 $-/-$ MEF monolayers cultured on collagen-coated slides were wounded by scraping with a pipet tip, allowed to migrate into the wound for 12 h, and then fixed and photographed. (C) Immortalized wild-type (open bars) and EDG-1 $-/-$ (filled bars) fibroblasts were allowed to migrate toward the indicated chemoattractants and chemotaxis was measured. (D) Migration defect is rescued by enforced expression of EDG-1. Immortalized EDG-1 $-/-$ fibroblasts were grown on collagen-coated slides and were transfected with vector (GFP) or with EDG-1-GFP, and after 16 h, migration into a wound was analyzed. The migration index is the ratio of the percentages of GFP-positive cells inside and outside the monolayer wounds from three random fields. Similar results were obtained in two additional experiments. Transfection efficiency was about 40%.

Figure 2. EDG-1 expression is not required for DNA synthesis stimulated by PDGF or SPP. (A) Wild-type (open bars) and EDG-1 $-/-$ (filled bars) MEFs were treated with PDGF-BB (20 ng/ml), serum (10 % v/v), sphingosine (Sph, 10 μ M), or SPP (10 μ M) and DNA synthesis was measured. Data are expressed as fold stimulation and are means \pm S.D. from three independent experiments. [3 H]Thymidine incorporation in

unstimulated EDG-1 +/+ and -/- MEFs was 750 ± 70 and 1100 ± 100 cpm per well, respectively. **(B)** Immortalized wild-type (open bars) and two clones of EDG-1 -/- fibroblasts (filled and stippled bars) were treated with the indicated agents and DNA synthesis was measured. **(C) *Edg-1* deletion abolishes PDGF-induced activation of p38 but not of ERK.** Wild type and EDG-1 -/- MEFs were serum-starved for 24 h, and then treated without or with PDGF-BB (20 ng/ml) for the indicated times. p38 and ERK activation was determined by Western blot analysis with phospho-specific anti-ERK1/2 and p38 antibodies. Blots were then stripped and re-probed with p38 antibody to demonstrate equal loading.

Figure 3. *Edg-1* disruption has no effect on survival. (A) *Edg-1* deletion does not alter susceptibility of MEFs to apoptotic stimuli. Subconfluent wild-type (open bars) and EDG-1 -/- MEFs (filled bars) were cultured in serum-free medium for 48 h, in the absence (Control) or presence of SPP (10 μ M), dihydro-SPP (10 μ M), or in the presence of 10 % serum with doxorubicin (DOXO, 1 μ g/ml) alone or with SPP (10 μ M), and apoptosis was determined. Data are means \pm S.E. of four independent experiments, each analyzed in triplicate. **(B)** Note the typical condensed fragmented nuclei of apoptotic cells (arrows) visualized by Hoechst staining in wild type and EDG-1 -/- fibroblasts after serum deprivation. **(C) The survival effects of PDGF and SPP are independent of EDG-1 expression.** Immortalized wild-type (open bars) and EDG-1 -/- (filled bars) cell lines were cultured in serum-free medium in the absence or presence of SPP (10 μ M), dihydro-SPP (10 μ M), PDGF (20 ng/ml), serum (10%) or doxorubicin (DOXO, 1 μ g/ml) in the absence or presence SPP (10 μ M), or with TNF- α (1 ng/ml) and

actinomycin D (ActD, 0.3 μ g/ml) in the absence or presence SPP (10 μ M). Apoptosis was measured after 48 h.

Figure 4. Changes in cytoskeletal architecture and focal contacts of EDG-1 null MEFs in response to PDGF. Wild type (A-D) and EDG-1 $-/-$ MEFs (E-H) were grown on cover slips for 24 h in serum-free medium and then stimulated without (A, B, E, F) or with 20 ng/ml PDGF-BB (C, D, G, H) for 20 min. Cells were then fixed, permeabilized and stained with phalloidin to detect actin fibers (green) and adhesion complexes were visualized with antibody to vinculin (red).

Figure 5. Enforced expression of EDG-1 restores PDGF-induced lamellipodial formation. Untransfected, immortalized EDG-1 null fibroblasts (A,B) or cells transiently transfected with GFP-EDG-1 (C-F), were serum starved overnight, and stimulated without (A, C, E) or with 20 ng/ml PDGF (B, D, F) for 5 min. Cells were fixed, permeabilized in 0.1% Triton X-100 for 6 min, and stained with Texas-red-phalloidin to detect actin fibers. Cells were examined by fluorescence confocal microscopy to localize the GFP-EDG-1 fusion protein (E, F) and actin cytoskeletal structures (C,D). The arrows indicate membrane ruffles. Note that cells not expressing GFP-EDG-1 (A, B), do not extend lamellipodia in response to PDGF.

Figure 6. Translocation of SPHK1 to membrane ruffles induced by PDGF. NIH 3T3 fibroblasts (A) and immortalized wild type murine fibroblasts (B) were transiently transfected with SPHK1-GFP, serum starved overnight, and stimulated without or with

20 ng/ml PDGF for 5 min as indicated. Cells were fixed, permeabilized and stained with Texas red-phalloidin to detect actin fibers then examined by fluorescence confocal microscopy to localize the SPHK1-GFP fusion protein (green) and actin cytoskeletal structures (red). The arrows indicate membrane ruffles.

Figure 7. *Edg-1* deletion causes aberrant FAK tyrosine phosphorylation and

activation. (A) Wild type and EDG-1 ^{-/-} MEFs were serum starved for 24 h, and then treated without or with PDGF-BB (20 ng/ml) for the indicated times. Equal amounts of cell lysate proteins were analyzed by Western blotting with anti-phosphotyrosine antibody. Blots were then stripped and re-probed with anti-FAK [pY⁵⁷⁷]. **(B, C)**

Abnormal focal adhesion complexes. Cells were treated with PDGF for the indicated time periods and Triton X-100 insoluble proteins analyzed by Western blotting with anti-FAK [pY⁵⁷⁷] phosphospecific antibodies and then re-probed with anti-FAK pan antibody (B) or with anti-paxillin monoclonal antibody and anti-CAS monoclonal antibody (C).

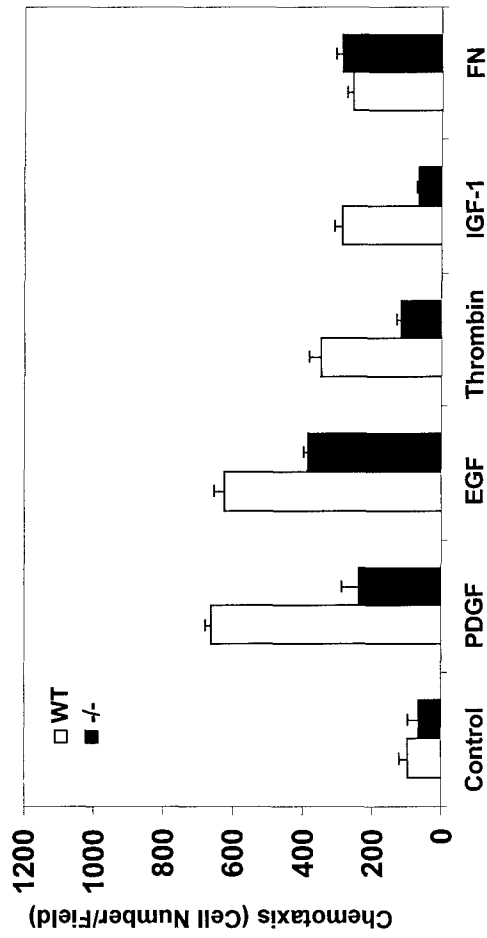
Figure 8. *Edg-1* deletion markedly reduces PDGF-induced Src activation. Wild

type and EDG-1 ^{-/-} MEFs **(A)** or immortalized fibroblasts **(B)** were serum-starved for 24 h, and then treated with PDGF-BB (20 ng/ml) for the indicated times and Triton X-100 insoluble fractions analyzed by Western blotting with anti-Src [pY⁴¹⁸] phosphospecific antibodies. Blots were then stripped and re-probed with anti-Src pan antibodies to demonstrate equal loading. **(C) Pertussis toxin and *edg-1* disruption suppresses** PDGF-stimulated activation of Src. Immortalized wild-type cells were serum-starved for 24 h, and then treated without (Cont.) or with PDGF-BB for 5 min. Where indicated,

cells were pre-treated with 200 ng/ml pertussis toxin for 2 h. Cells were lysed in RIPA buffer and immunoprecipitated with Src, Yes or Fyn antibodies. Immunoprecipitates were washed and in vitro kinase activity determined. Tyrosine kinase activities are expressed as means \pm S.D. of ^{32}P cpm incorporated into a synthetic peptide substrate.

Inset: Immortalized wild-type and EDG-1 $-/-$ cell lines were serum-starved for 24 h, treated without or with PDGF-BB for 5 min and lysates immunoprecipitated and assayed for Src and Fyn activity. Data are expressed as means of fold increases in activity compared to unstimulated cells.

A



C

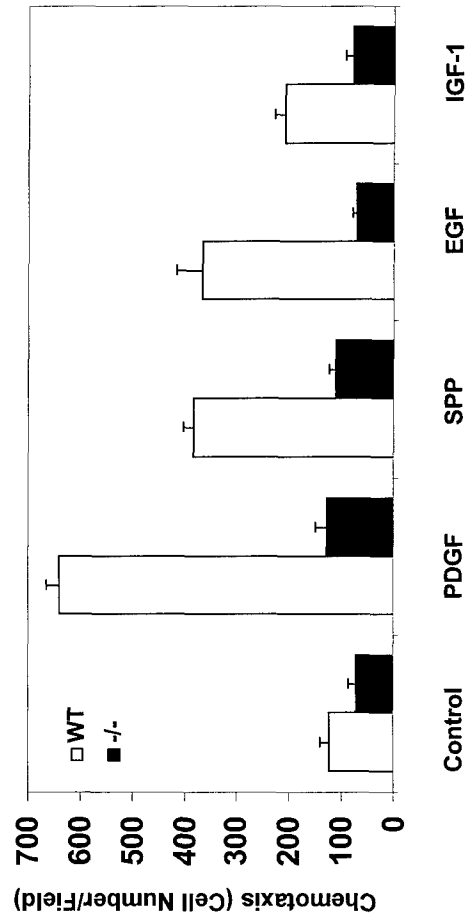
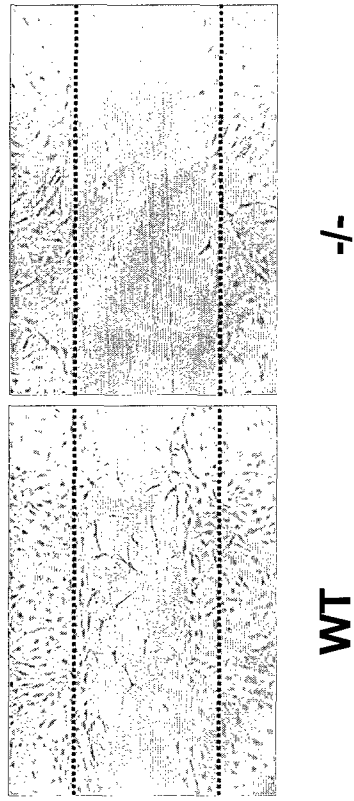
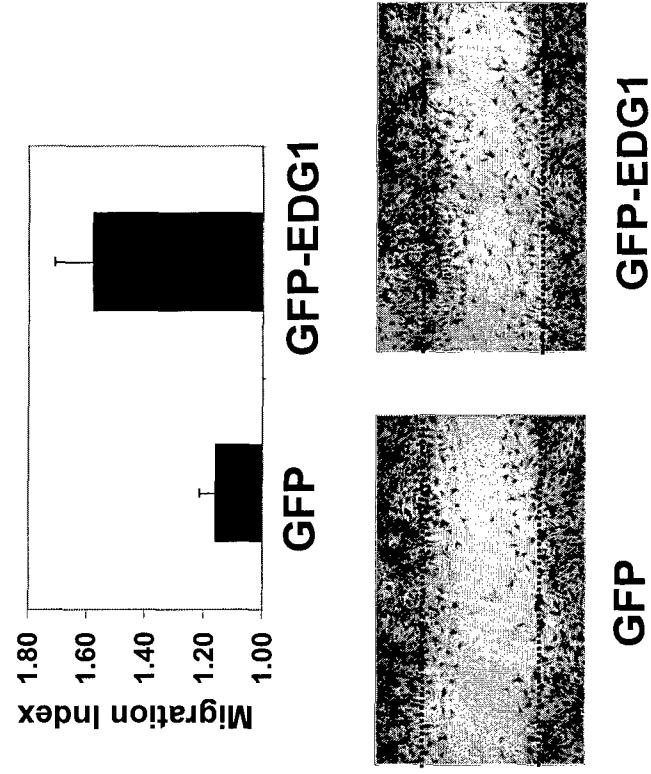


Figure 1

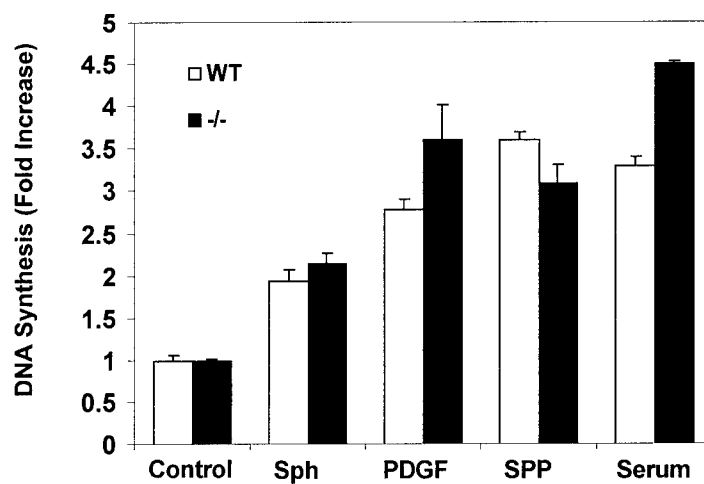
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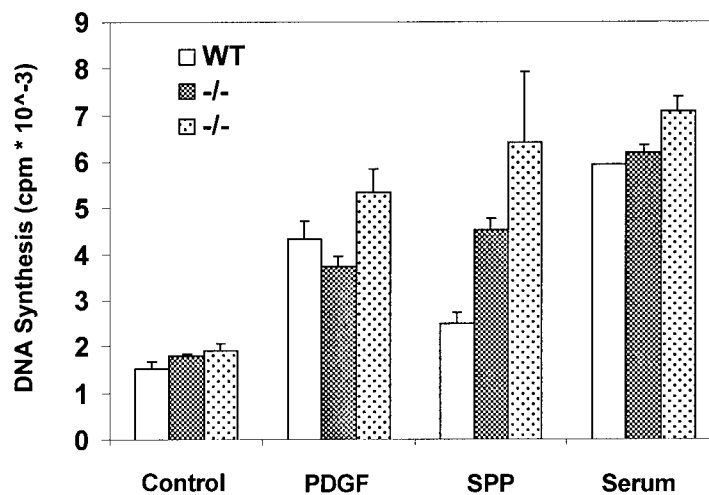
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A



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C

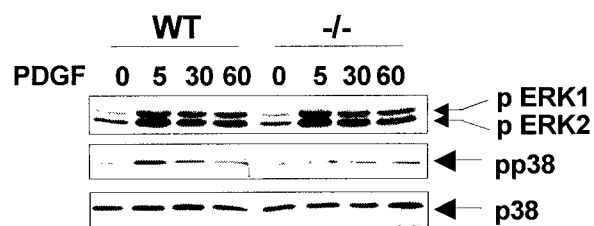
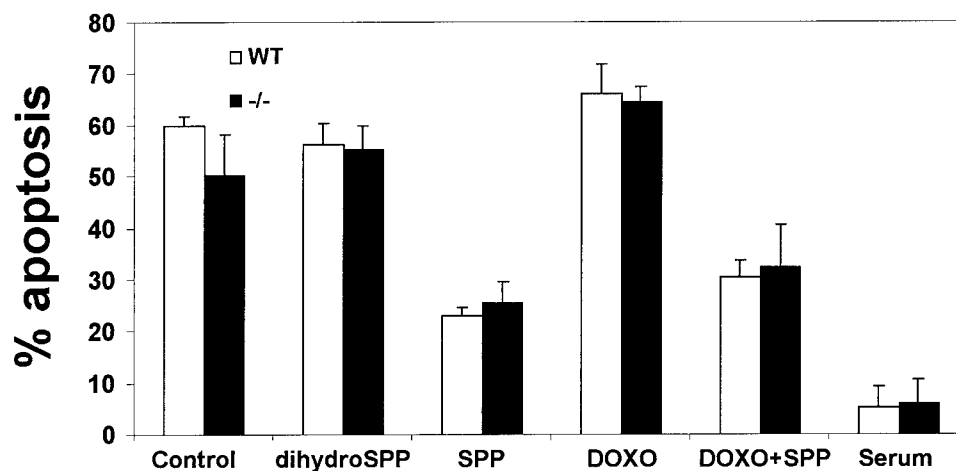
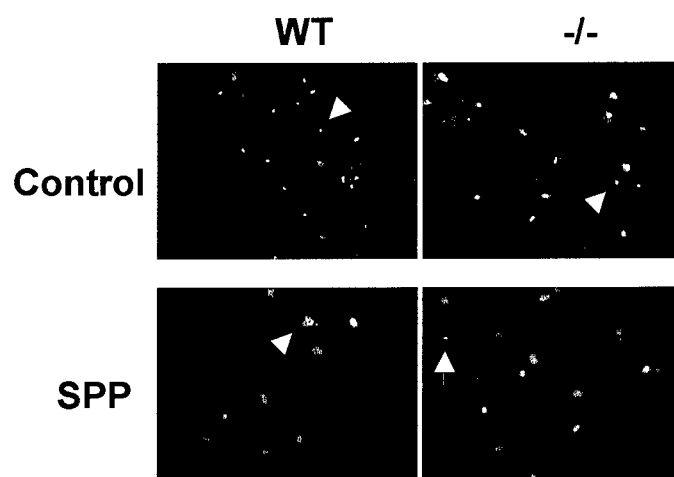


Figure 2

A



B



C

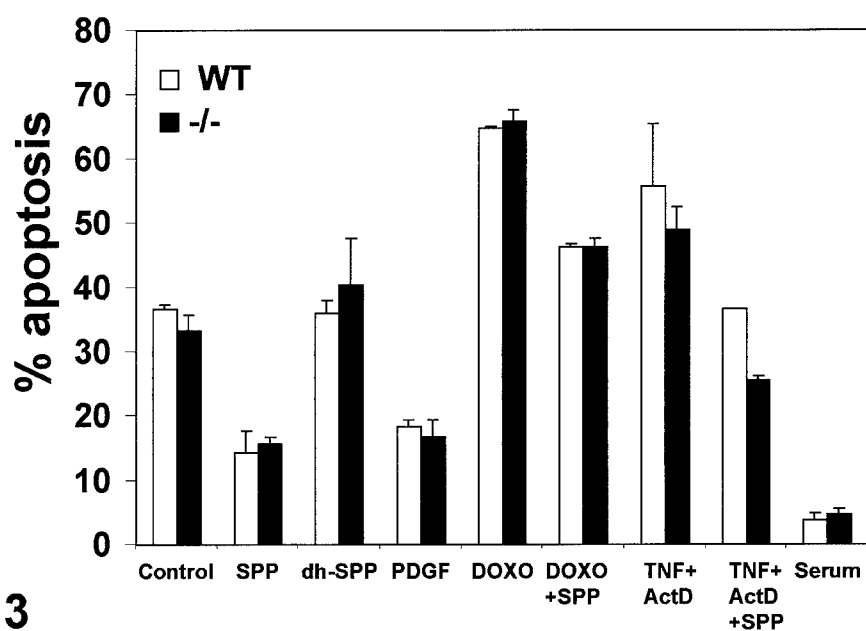


Figure 3

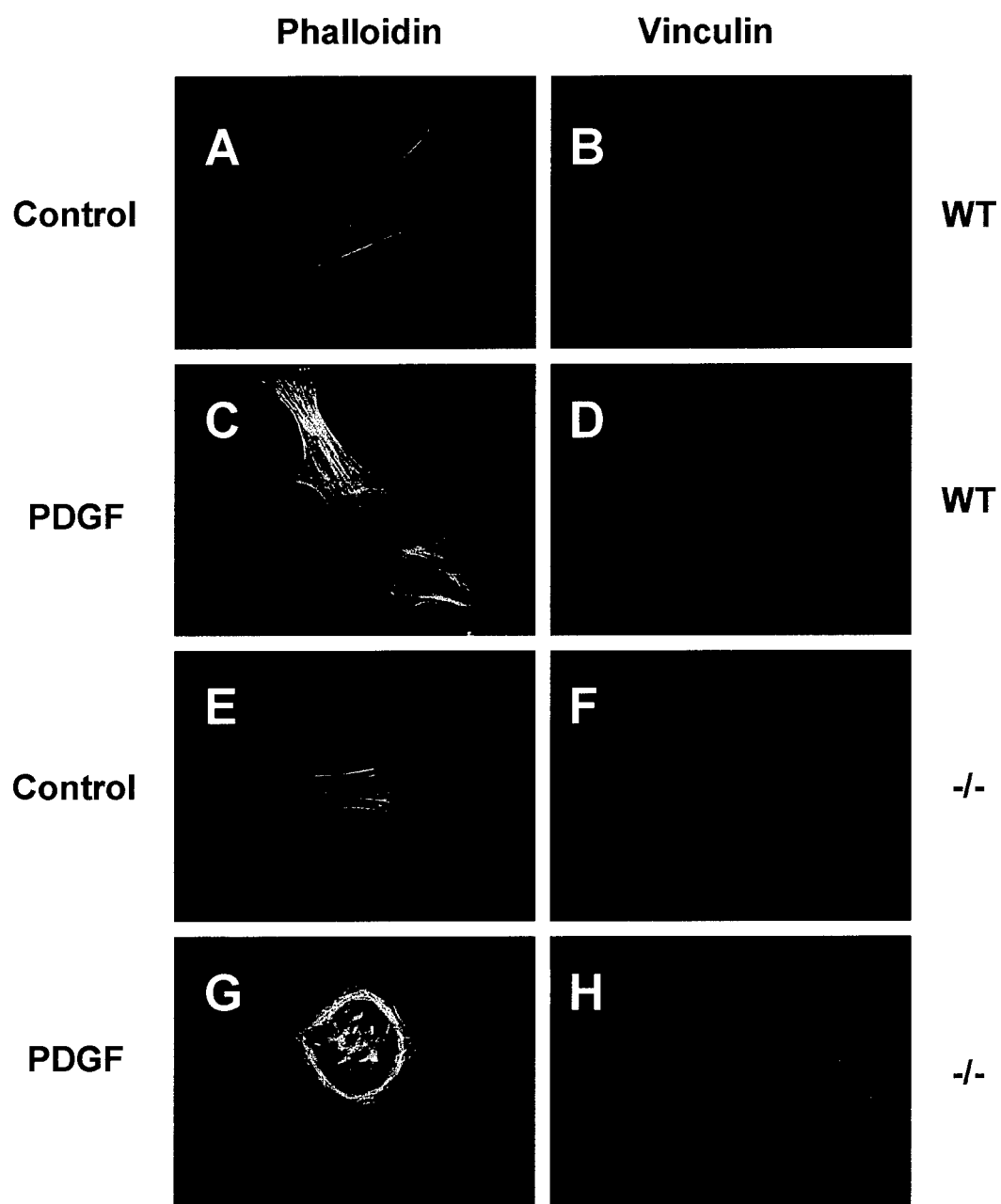


Figure 4

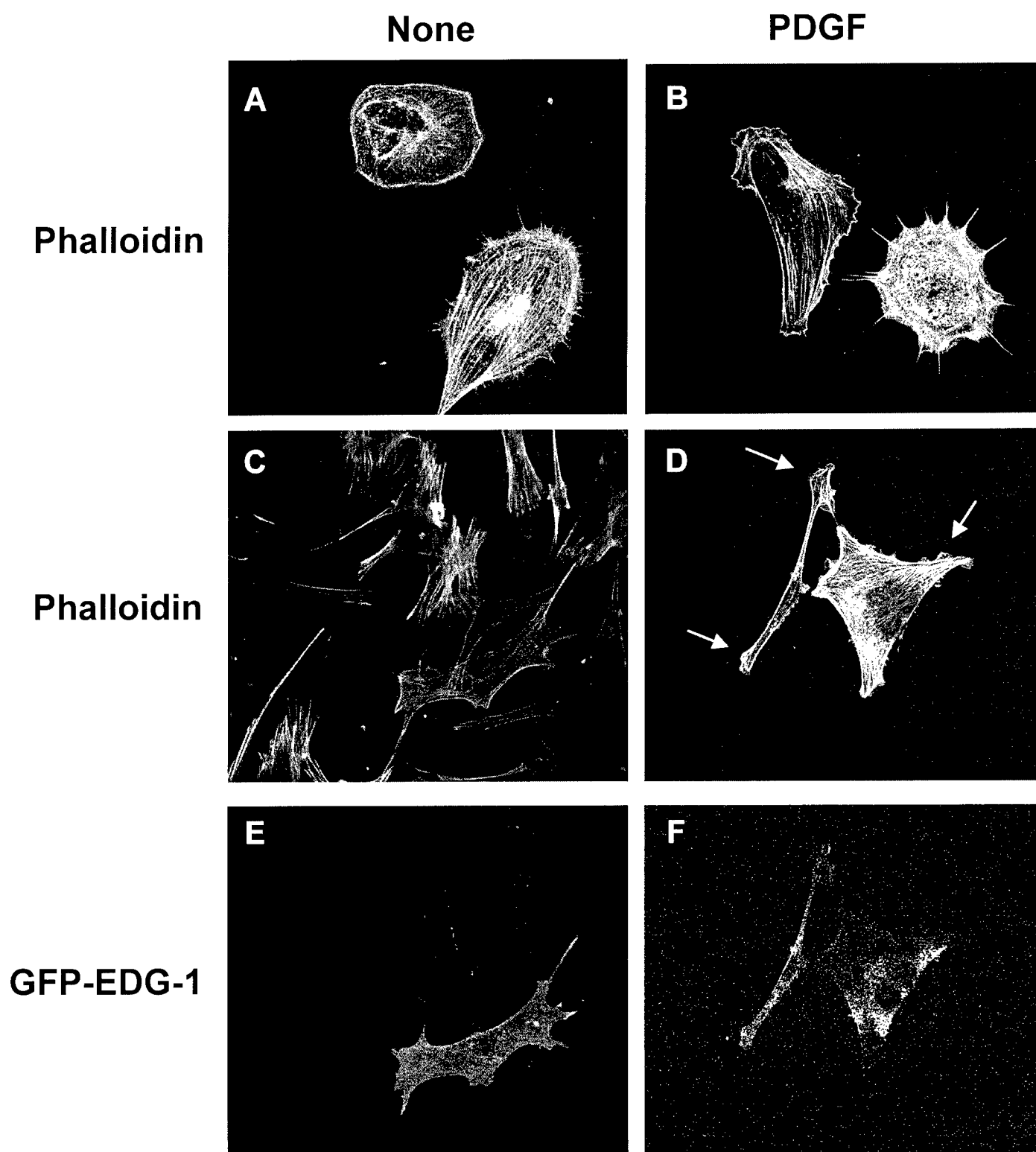
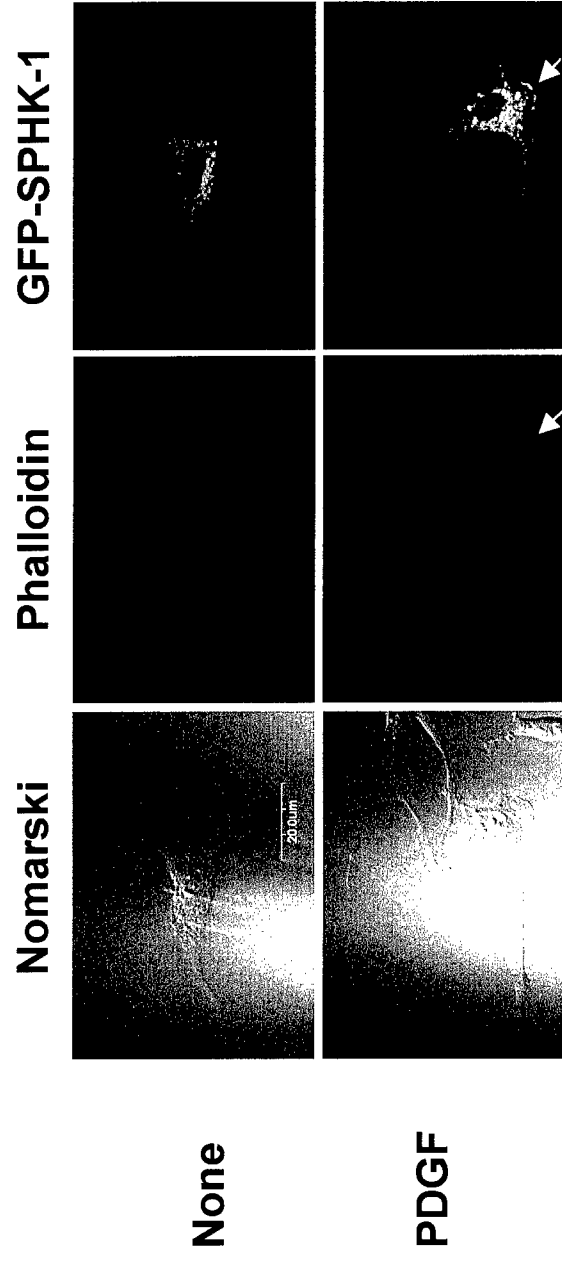


Figure 5

A.



B.

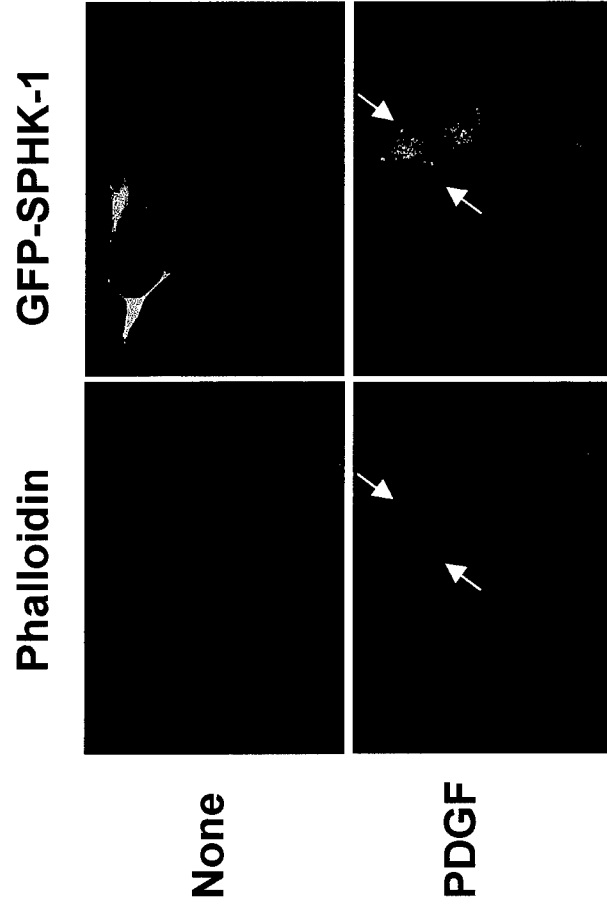
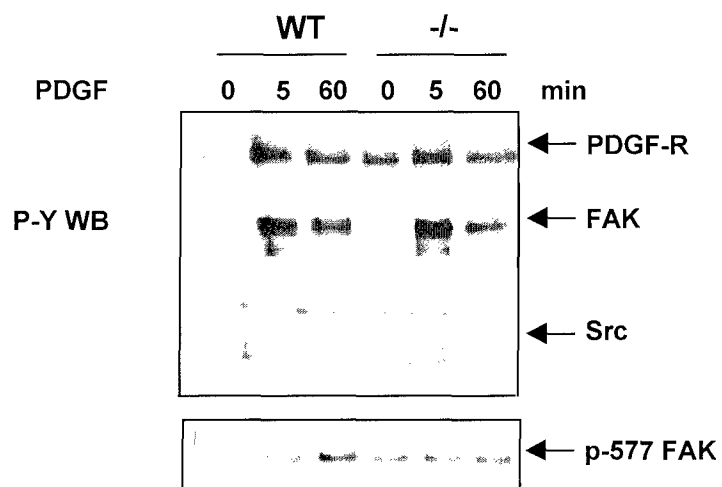
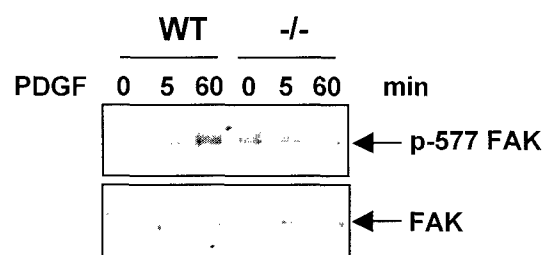


Figure 6

A



B



C

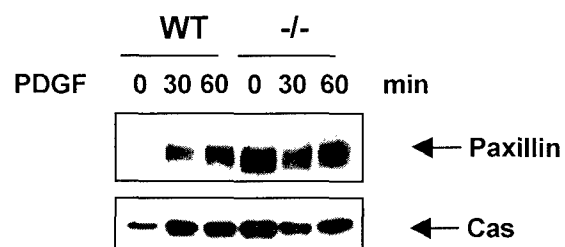
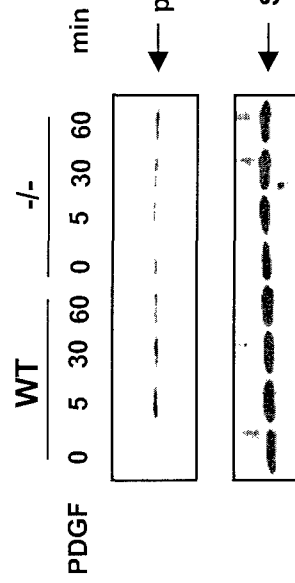
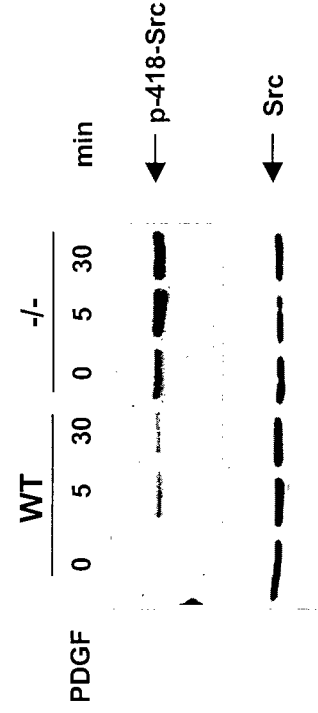


Figure 7

A



B



C

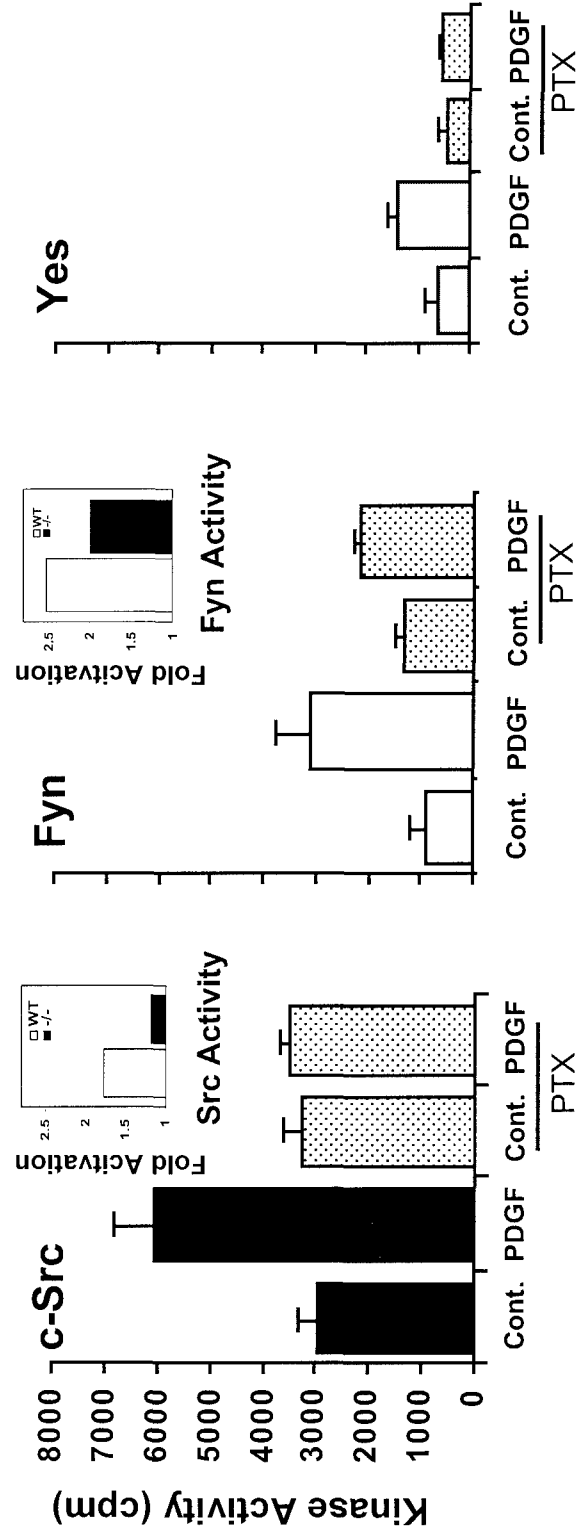


Figure 8

**SPHINGOSINE KINASE TYPE 1 PROMOTES ESTROGEN-DEPENDENT
TUMORIGENESIS OF BREAST CANCER MCF-7 CELLS**

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Key Words: Sphingosine-1-phosphate, breast cancer

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Institutes of Health (S.S). V.N. was supported by a postdoctoral fellowship and J.P.H. by a
predoctoral fellowship from the United States Army Medical Research and Materiel Command.

²**The abbreviations used are:** BSA, bovine serum albumin; DMS, N,N-dimethylsphingosine;
EDG, endothelial differentiation gene; IMEM, Richter's Improved Minimal Essential Medium;
FCM, fibroblast conditioned medium; GFP, green fluorescent protein; GPCR, G protein-coupled
receptors; dihydro S1P, sphinganine-1-phosphate; LPA, lysophosphatidic acid; PDGF, platelet-
derived growth factor; S1P, sphingosine-1-phosphate; FBS, fetal bovine serum.

ABSTRACT

The sphingolipid metabolite, sphingosine-1-phosphate (S1P) formed by phosphorylation of sphingosine, has been implicated in cell growth, suppression of apoptosis, and angiogenesis. We have examined whether increased intracellular S1P produced by overexpression of sphingosine kinase contributes to tumorigenesis of breast adenocarcinoma MCF-7 cells. Sphingosine kinase type 1 (SPHK1) blocks MCF-7 cell death induced by anti-cancer drugs, sphingosine and TNF- α . The cytoprotective effect of SPHK1 was reversed by N,N-dimethylsphingosine, a specific inhibitor of SPHKs. Enforced expression of SPHK1 in MCF-7 cells also conferred a growth advantage as determined by proliferation and growth in soft agar. Although no changes in estrogen receptor levels could be detected, estrogen antagonists eliminated this growth advantage. Both ERK and Akt have been implicated in MCF-7 cell growth. However, SPHK1 overexpression in MCF-7 cells stimulated ERK1/2 but had no effect on Akt. In most cell types, the mitogenic effects of S1P stem from binding to specific cell surface. Surprisingly, parental MCF-7 cells exhibit only weak mitogenesis in response to either S1P or dihydroS1P, ligands for S1P receptors, and SPHK1/MCF-7 cells did not release detectable amounts of S1P into the medium. When injected into mammary fat pads of ovariectomized nude mice implanted with estrogen pellets, MCF-7 cell expressing SPHK1 formed more and larger tumor than vector transfectants. There was also higher microvessel density in the periphery of these tumors. Collectively, our results suggest that SPHK1 may play an important role in breast cancer progression by regulating tumor cell growth and survival. SPHK inhibition might be a useful adjunct to anti-estrogen treatment of breast cancer.

INTRODUCTION

Sphingolipid metabolites ceramide, sphingosine, and sphingosine-1-phosphate (S1P) play an important role in regulation of cell proliferation, survival, and cell death (reviewed in (Kolesnick and Hannun, 1999 *Trends Biochem. Sci.* **24**, 224-225; Merrill *et al.*, 1997 *Toxicology and Applied Pharmacology* **142**, 208-225; Spiegel and Milstien, 2000 *FEBS Lett.* **476**, 55-67.)). Ceramide and sphingosine usually inhibit proliferation and promote apoptosis (Kolesnick and Hannun, 1999 *Trends Biochem. Sci.* **24**, 224-225; Merrill, *et al.*, 1997 *Toxicology and Applied Pharmacology* **142**, 208-225.), while the further metabolite S1P stimulates growth and suppresses ceramide mediated apoptosis (Spiegel and Milstien, 2000 *FEBS Lett.* **476**, 55-67.). It has been suggested that the dynamic balance between levels of the sphingolipids metabolites, ceramide and sphingosine versus S1P, and consequent regulation of opposing signaling pathways, is an important factor that determines the fate of cells (Cuvillier *et al.*, 1996 *Nature* **381**, 800-803.). This has important clinical implications including prevention of infertility in female cancer patients, as increased S1P or decreased ceramide can prevent radiation-induced oocyte loss (Morita *et al.*, 2000 *Nature Med.* **6**, 1109-1114; Perez *et al.*, 1997 *Nature Med.* **3**, 1228-1232.). The relevance of this “sphingolipid rheostat” and its role in regulating cell fate has been borne out by work in many labs using many different cell types and experimental manipulations (reviewed in (Spiegel and Milstien, 2000 *FEBS Lett.* **476**, 55-67.)). A central finding of these studies is that sphingosine kinase (SPHK), the enzyme that phosphorylates sphingosine to form S1P, is a critical regulator of the sphingolipid rheostat, as it not only produces the pro-growth, anti-apoptotic messenger S1P, but also decreases levels of pro-apoptotic ceramide and sphingosine. SPHK is activated by numerous external stimuli, among which growth and survival factors are prominent (reviewed in (Spiegel and Milstien, 2000 *FEBS Lett.* **476**, 55-67.)). Enforced expression of SPHK1 in NIH 3T3 fibroblasts increased the proportion of cells in S phase of the cell cycle by promoting the G₁-S transition; it also reduced the doubling time of these cells, an effect that was especially marked under low-serum conditions, indicating that intracellular S1P may be an important regulator of cell growth (Olivera *et al.*, 1999 *J. Cell Biol.* **147**, 545-558.). Furthermore, SPHK1 expression also resulted in acquisition of a transformed phenotype, as determined by assays of focus formation, colony growth in soft agar, and the ability to form tumors in NOD/SCID mice, suggesting that the corresponding wild-type gene may act as an oncogene (Xia *et al.*, 2000 *Curr. Biol.* **10**, 1527-

1530.). With the use of a SPHK inhibitor and a dominant negative mutant of this enzyme, Xia *et al.* (Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.) also showed that SPHK contributes to cell transformation mediated by oncogenic H-Ras.

Overexpression of SPHK1 also protected against apoptosis induced by serum deprivation or by ceramide in NIH 3T3 fibroblasts (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558.), and PC12 cells. In the latter cells, the anti-apoptotic effect correlated with inhibition of caspases-2, -3, and -7 and of the stress-activated protein kinase, JNK (Edsall *et al.*, 2001 *J. Neurochem.* **76**, 1573-1584.). Collectively, these observations implicate SPHK1 and S1P formation in cell growth, transformation, and cancer.

S1P regulates growth by two mechanisms of action that are not necessarily mutually exclusive (Van Brocklyn *et al.*, 1998 *J. Cell Biol.* **142**, 229-240.). S1P binds to G-coupled protein receptors of the Edg-family (now known as S1PRs) and induces cell growth, migration, survival, and angiogenesis (reviewed in [Pyne, 2000 #4193; Spiegel, 2000 #3964; [Goetzl, 2001 #4251; Hla, 2001 #4476]. Intracellularly, it binds to unidentified receptors and induces calcium mobilization [Meyer zu Heringdorf, 1999 #4352], mitogenesis (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558; Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.) and survival (Cuvillier, et al., 1996 *Nature* **381**, 800-803; Morita, et al., 2000 *Nature Med.* **6**, 1109-1114; Perez, et al., 1997 *Nature Med.* **3**, 1228-1232.). Understanding of the intra- and extra-cellular actions of S1P are further complicated by stimulation of SPHK by binding of S1P to its receptors [Meyer zu Heringdorf, 2001 #4386], and conversely, endogenously produced S1P is able to activate EDG-1/S1P₁ in an autocrine or paracrine manner [Hobson, 2001 #4113; Rosenfeldt, 2001 #4380]. Regardless of the precise mechanism of action, S1P could also be involved in tumorigenesis not only because it promotes cell survival and proliferation, but also because it stimulates angiogenesis (English *et al.*, 2000 *FASEB J.* **14**, 2255-2265; Lee *et al.*, 1999 *Cell* **99**, 301-312; Lee *et al.*, 1999 *Biochem. Biophys. Res. Commun.* **264**, 743-750; Wang *et al.*, 1999 *J. Biol. Chem.* **274**, 35343-35350.) and regulates vascularization [Liu, 2000 #4418; Hobson, 2001 #4113; Garcia, 2001 #4413].

Previously it has been shown that S1P induces proliferation of human breast carcinoma MCF-7 cells (Goetzl *et al.*, 1999 *Cancer Res.* **59**, 4732-4737.). In addition, in MCF-7 cells, overexpression of SPHK1 inhibited chemotactic motility compared with vector-transfected cells and markedly increased cellular S1P levels in the absence of detectable secretion (Wang *et al.*, 1999 *Cancer Res.* **59**, 6185-6191.). These results suggest that the inhibitory effect of SPP on

chemotactic motility of human breast cancer cells is likely mediated through intracellular actions of S1P rather than through cell surface receptors. In this report we examined whether SPHK1 contributes to tumorigenesis of breast cancer MCF-7 cells ex vivo and in nude mice. We found that overexpression of SPHK1 confers a growth advantage to MCF-7 cells by an estrogen-dependent mechanism related to activation of extracellular signal-related kinases (ERK) and furthermore promotes tumorigenesis of these cells in nude mice and neovascularization of the tumors.

MATERIALS AND METHODS

Materials. S1P, dihydroS1P, sphingosine and N,N-dimethylsphingosine (DMS) were from Biomol Research Laboratory (Plymouth Meeting, PA). Medium, serum, penicillin/streptomycin and glutamine were from Biofluids (Rockville, MD). G418 sulfate was from Mediatech (Herndon, VA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), crystal violet, doxorubicin, cycloheximide and 17- β -estradiol were from Sigma (St. Louis, MO). ICI 182780 was from Tocris (Ballwin, MO). PD98059 was from Calbiochem (La Jolla, CA). UO126 was from Promega (Madison, WI). Agar was from DIFCO (Detroit, MI). TNF- α was from Roche Molecular Biochemicals (Indianapolis, IN).

Cell Culture. MCF-7 cells (passage 72 from Lombardi Cancer Center Cell Facility) transfected with vector alone (c-myc-pcDNA3) or with mSPHK1 were cultured as described previously (Wang, et al., 1999 *Cancer Res.* **59**, 6185-6191.), and were maintained in high glucose IMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine and 0.5 g/L G418.

Cell Death Assays. Cell viability was determined with the MTT dye reduction assay as described previously (Cuvillier, et al., 1996 *Nature* **381**, 800-803.). Results are expressed as percent dead cells relative to the untreated control at the indicated time. Briefly, cells (4×10^3 /well in 100 μ l) were plated in 96-well plates. The media was changed after 24 h to serum-free, phenol red-free medium and cells treated with the indicated agents as described in figure legends. At various times after treatment, cells were incubated for 4 h after addition of 10 μ l of

MTT solution to a final concentration of 0.5 mg/ml. Formazan dye was solubilized for 16 h by addition of 100 µl of 20% SDS in 0.01M HCl, and quantitated in a microplate reader at a wavelength of 570 nm minus 650 nm. In some experiments, cell viability was determined by trypan blue exclusion after collecting adherent and floating cells.

Immunoblotting. Cells were harvested in buffer containing 10mM HEPES (pH=7.4), 2 mM EDTA, 0.1 % (w/v) CHAPS, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin A, 10 µg/ml aprotinin, and 20 µg/ml leupeptin. After centrifugation at 10,000 x g for 10 min at 4 C, proteins in the supernatants was quantitated by Coomassie Plus Assay (Pierce, Rockford, IL) and analyzed by SDS/PAGE. Proteins were transferred to nitrocellulose and probed with specific antibodies: clone 7D3-6 mouse monoclonal anti-PARP 1:1000 (PharMingen, Hamburg, Germany); rabbit polyclonal anti-Bax 1:1000 (PharMingen); rabbit polyclonal anti-Caspase-7 1:1000 (Oncogene, Darmstadt, Germany); rabbit polyclonal anti-phospho-JNK 1:1000 (New England Biolabs, Beverly, MA); rabbit polyclonal anti-JNK 1:1000 (New England Biolabs); rabbit polyclonal anti-phospho-p44/42 MAPK 1:1000 (New England Biolabs); rabbit polyclonal anti-p44/42 MAPK 1:1000 (New England Biolabs); rabbit polyclonal anti-phospho-AKT 1:1000 (New England Biolabs); rabbit polyclonal anti-AKT1:1000 (New England Biolabs); and clone HD11 mouse monoclonal anti-cyclin D1 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA)

Cell Growth Assays. Cells were seeded in 96-well plates at 10% confluence (1000 cells per well) in media containing 10 % FBS. After attachment cells were washed with serum-free medium and placed in IMEM containing 0.5 % FBS, 10 % FBS or 5 % CCS. Additives (DMS, PD98059, UO126, estradiol and ICI) were replenished every 2 days by changing medium. Prior to addition of estradiol or ICI, cells were grown in phenol red-free IMEM supplemented with 5 % charcoal-stripped calf serum (CCS) for 2 days. At the indicated times, cells were stained with crystal violet as previously described (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558.). Incorporated dye was solubilized of in 100 µl sodium citrate (pH 4.2), 50 % ethanol and the absorbance was measured in at 570 nm. In some experiments, cell growth was assessed by counting cell in a hemocytometer after trypsinization.

Soft Agar Assays. Colony growth in soft agar was determined in 6-well plates prepared in triplicates overlaying 2×10^4 cells resuspended in a 0.35 % agar solution in IMEM with 4 % FBS on a lower layer of 0.6 % agar solution in IMEM containing 5 % FBS [Weber, 2000 #4500]. Colonies larger than 60 μm were scored at 7 and 10 days after preparation using an Ommicon 3600 Image Analysis system.

Estrogen Receptor Protein Assays. Cells were cultured in IMEM with 10 % FBS until 80 % confluent, and then placed in phenol red-free IMEM containing 5 % CCS. After 2 days, cells were treated with 1 nM estradiol, and harvested in high salt buffer (10 mM Tris, pH 7.4, 1.5 mM EDTA, 5 mM Na_2MoO_4 , 0.4 M KCl, 1 mM monothioglycerol, 2 mM leupeptin) followed by sonication. After centrifugation at $100,000 \times g$ for 1 h at 4 °C, protein concentration of the supernatants was determined by the bicinchoninic acid assay (Pierce). Estrogen receptor- α levels were determined by immunoassays (Abbott Laboratories, North Chicago, IL).

Cell Cycle Analysis. Single cell suspensions were prepared by trypsinization and after washing twice with PBS, cell pellets were resuspended in 40 mM citrate buffer (pH 7.6) containing 250 mM sucrose and 5 % DMSO. Cellular DNA was stained with propidium iodide and cell cycle distribution was determined by flow cytometry (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558.).

Tumor Growth in Nude Mice. Female ovariectomized 4 to 6 wk old BALB/c nu/nu mice were purchased from Charles River Laboratory, and implanted subcutaneously with 0.72 mg 17 β -estradiol pellets (Innovative Research, Rockville, MD) at the beginning of the experiment. Cells were grown until 80 % confluent as described above. One dish of MCF-7 vector or MCF-7/SPHK1 cells was trypsinized and cells counted by trypan blue exclusion. Cells were resuspended in IMEM (5×10^6 cells in 100 μl) and passed through an 18-gauge needle several times to eliminate clumps. Cells were injected into the mammary fat pads of the mice (McLeskey et al., 1993 *Cancer Res.* **53**, 2168-2177.). Tumor volumes were calculated by caliper measurements taken twice weekly. Mice were sacrificed 8 weeks post-injection and tumors were cut in half. One half was fixed in 10% buffered formalin and processed histologically. The other half was used to confirm transgene expression using the sphingosine kinase assay as described previously (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558.). The results were

analyzed by defining a differentiation curve of the difference in tumor growth between vector and SPHK1 cells as a function of time for each mouse. The generalized estimating equation method was performed by SAS PROC GLM.

RESULTS

Sphingosine Kinase Overexpression Enhances Survival of MCF-7 Cells

Tumor progression depends on a balance between cell survival and cell proliferation. Apoptosis of non-transformed NIH 3T3 fibroblasts is reduced by overexpression of SPHK1 (Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558; Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.). However, much less is known of the role of SPHK1 in apoptosis of cancer cells. We previously found that breast adenocarcinoma MCF-7 cells overexpressing murine SPHK1 (MCF-7/SPHK1) have elevated intracellular S1P levels but are unable to release S1P to the extracellular milieu (Wang, et al., 1999 *Cancer Res.* **59**, 6185-6191.). We have now examined the effects of various stress stimuli on growth and survival of these cells. MCF-7/SPHK1 cells are more resistant than control cells to death induced by the antineoplastic agent doxorubicin, or TNF- α in the presence of actinomycin D, which sensitizes cells to the toxic effect of TNF- α (Fig. 1A, B). Previously, we have shown that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin and itself induces apoptosis in MCF-7 cells [Cuvillier, 2001 #4340]. In agreement, sphingosine markedly induced apoptosis of MCF-7 cells and there was significantly less apoptosis in sphingosine treated MCF-7/SPHK1 cells (Fig. 1C), indicating that increased intracellular S1P levels protect MCF-7 cells against sphingosine-induced apoptosis. Further confirming that the cytoprotective effect was due to the enzymatic activity of SPHK1, N,N-dimethylsphingosine (DMS), a potent competitive SPHK inhibitor (Edsall *et al.*, 1998 *Biochemistry* **37**, 12892-12898.), reduced the apoptosis sparing effect (Fig. 1D). In contrast to NIH 3T3 fibroblasts, in which serum deprivation markedly induced cell death that was abrogated by enforced SPHK1 expression, MCF-7 cells are not sensitive to serum withdrawal (Fig. 1E).

Effect of SPHK1 Overexpression on Activation of Caspases and JNK

To examine whether cell death modulated by SPHK1 was due to inhibition of a caspase-dependent program, the activation of the caspases that drive the effector phase of apoptosis by

cleaving key proteins, was assessed. MCF-7 cells do not exhibit typical nuclear apoptotic changes during apoptosis since they are deleted of caspase-3 and depend mainly on caspase-7 for the execution of cell death [Janicke, 1998 #2795; Cuvillier, 2001 #4340]. Activation of caspase-7 and the consequent cleavage of PARP were delayed by overexpression of SPHK1 during apoptosis induced by doxorubicin, DMS (Fig. 2) or Sph (data not shown). It should be pointed out that the effect of SPHK1 overexpression on caspase-7 activation did not correlate with the protection conferred by SPHK1 (Fig. 1) and raises the possibility that S1P might inhibit other anti-apoptotic pathways.

Apoptosis in MCF-7 cells involves activation of a mitochondrial pathway modulated by Bcl-2 family members [Scaffidi, 1998 #2696; Cuvillier, 2001 #4340]. This pathway is initiated in part through phosphorylation and activation of JNK and subsequent stabilization of wild-type P53 [Fuchs, 1998 #4492]. P53 in turn induces transcription of the apoptosis-promoting protein Bax that then translocates to the mitochondria to promote cytochrome c release. Doxorubicin increased phosphorylation of JNK in MCF-7 cells within 48 h (Fig. 3). Reminiscent of previous results where overexpression of SPHK1 effectively inhibited activation of JNK in serum-deprived PC12 cells (Edsall, et al., 2001 *J. Neurochem.* **76**, 1573-1584.), overexpression of SPHK1 reduced phosphorylation of JNK in MCF-7 cells cultured in serum free medium. However, enforced expression of SPHK1 did not diminish doxorubicin-induced JNK phosphorylation (Fig. 3).

Recently, exogenous S1P was found to suppress cellular levels of Bax during cytokine-induced apoptosis of human T lymphoblastoma cells (Goetzl *et al.*, 1999 *J. Immunol.* **162**, 2049-2056.). In contrast, only a small decrease in Bax expression was observed in MCF-7/SPHK1 cells that were serum starved for 48 h (Fig. 3). Bax levels were however reduced after doxorubicin treatment, consistent with reports of Bax proteolysis during apoptosis [Thomas A Oncogene 1996 ?]. No differences in levels of Bcl-2 or phospho-BAD between MCF-7/SPHK1 and vector cells were detected by immunoblotting after serum deprivation (data not shown). Taken together these results suggest that neither Bax nor JNK play important roles in the anti-apoptotic effect of SPHK1.

Sphingosine Kinase-1 Overexpression Confers Growth Advantage to MCF-7 Cells

Because the most well known biological effect of S1P is stimulation of proliferation, we next examined the effect of enforced expression of SPHK1 on growth of MCF-7 cells. Overexpression of SPHK1 enhanced proliferation of MCF-7 cells in the presence of 10 % but not in 0.5 % fetal bovine serum (Fig. 4A). Analyses of the growth curves during the exponential growth phase revealed that SPHK1 expression decreased the doubling time from 37.7 ± 3.3 h to 28.0 ± 2.9 h. The growth advantage of MCF-7/SPHK1 was obliterated in a dose-dependent manner by DMS, suggesting that the proliferation stimulating effect of SPHK1 depends on its enzymatic activity (Fig. 4B). Moreover, FACS analysis revealed that even in the presence of 10% serum, which markedly increased the proportion of cells in the S phase and G₂/M phase, overexpression of SPHK1 further reduced the fraction of cells in G₀/G₁, increased the proportion in the G₂/M phase, and to a lesser extent, in the S phase (Table I). This data suggests that either a greater proportion of SPHK1 transfected cells are cycling and/or that the duration of the G₁ phase is shortened compared to vector transfected cells.

Previously, it has been shown that NIH 3T3 fibroblasts overexpressing SPHK1 acquire a transformed phenotype as determined by anchorage-independent growth in soft agar assays (Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.). Similarly, MCF-7/SPHK1 cells formed more and larger colonies than vector cells when grown in agar in the presence of serum (Fig. 4C). However, no colonies were formed when MCF-7/SPHK1 cells were plated in serum-free agar (data not shown), suggesting that factors present in serum are required for SPHK1-induced transformation.

The mitogenic effects of S1P stem from binding to specific cell surface receptors [Goetzl, 1998 #2831; Hla, 2001 #4476] or to intracellular actions (Pyne and Pyne, 2000 *Biochem. J.* **349**, 385-402.; Spiegel and Milstien, 2000 *Biochim. Biophys. Acta* **1484**, 107-116.) depending on the cell type and expression of receptors. Previously, it has been reported that MCF-7 cells proliferate in response to the addition of S1P through activation of EDG-3/S1P₂ and EDG-5/S1P₃ expressed on these cells (Goetzl, et al., 1999 *Cancer Res.* **59**, 4732-4737.). However, intracellular levels of S1P were elevated in MCF-7/SPHK1 cells but no measurable release into the media could be detected (Wang, et al., 1999 *Cancer Res.* **59**, 6185-6191.). DihydroS1P also binds to and stimulates all of the S1PRs but in contrast to S1P has no known second messenger action and thus is useful to differentiate between receptor-driven effects and intracellular actions. At nanomolar concentration, neither S1P nor dihydroS1P induced significant proliferation of parental MCF-7 cells cultured in 5 % CCS (Fig. 4D (should be Fig. 5A), whereas overexpression

of SPHK1 still enhanced growth in these conditions, albeit not as effectively as in 10% FBS (Fig. 5B). Although previously we and others have found that S1P also induced a moderate increase in DNA synthesis of MCF-7 cells as measured by [3 H]thymidine incorporation (Goetzl, et al., 1999 *Cancer Res.* **59**, 4732-4737; Wang *et al.*, 1999 *Exp. Cell Res.* **247**, 17-28.), no significant increase in cell numbers was detected even when MCF-7 cells were cultured for 8 days in 10% FBS containing S1P or dihydroS1P. It was previously suggested that exogenous S1P enhanced growth of MCF-7 cells partly by enhancing secretion of type II insulin-like growth factor (IGF-II) (Goetzl, et al., 1999 *Cancer Res.* **59**, 4732-4737.), as monoclonal anti-IGF-II and anti-IGFR1 antibodies strongly suppressed proliferation induced by S1P. However, in the presence of serum where overexpression of SPHK1 significantly enhanced cell growth, addition of anti-IGF-IR antibody which blocks the effects of both IGF-I and IGF-II, did not have a marked effect on proliferation during eight days of culture. Collectively, these results suggest that the mitogenic effect of SPHK1 and intracellularly generated S1P might not be due to activation of S1P receptor signaling present in MCF-7.

SPHK1 Stimulation of MCF-7 Cell Growth is Estrogen Dependent

MCF-7 cells have functional estrogen receptors and are dependent on estrogen for growth. In agreement with many previous studies (reviewed in [Lippman, 2001 #4494]), 17- β estradiol markedly stimulated MCF-7 cell proliferation which was further enhanced by SPHK1 overexpression (Fig. 5A should be 5C). In addition, the mitogenic advantage induced by SPHK1 was reduced in the absence of estrogen when the cells were grown in 5 % CCS, and was ablated by the pure estrogen antagonist ICI 182,780 (Fig. 5B should be 5D), suggesting that estrogen signaling is important for the mitogenic effect of SPHK1. Expression of estrogen receptor- α determined by immunoassay was not significantly different from the vector transfected cells in MCF-7/ SPHK1 (Table II) and there were no differences in [3 H]-estradiol binding (data not shown). Conversely, treatment of MCF-7 cells with estrogen did not activate SPHK1 ruling out the possibility that estrogenic stimulation results in the production of S1P. Collectively, these results suggest that estrogen and SPHK1 activate distinct potentially complementary signaling pathways that ultimately converge to stimulate cell growth.

Sphingosine Kinase Overexpression in MCF-7 Cells Stimulates Erk But Has No Effect on Akt

To gain insight into the proliferative effect of SPHK, we examined signaling pathways that have been implicated in MCF-7 cell growth: activation of the MAP kinase ERK 1/2 and stimulation of the phosphatidylinositol 3-kinase (PI3K) pathway, resulting in activation of the PKB/Akt family of serine/threonine protein kinases. Similar to 17- β estradiol, S1P activates ERK in diverse cell types in pertussis toxin-sensitive and -insensitive manners (reviewed in (Pyne and Pyne, 2000 *Pharmacol. Ther.* **88**, 115-131.)). In contrast to PC12 cells (Edsall, et al., 2001 *J. Neurochem.* **76**, 1573-1584.), enforced expression of SPHK1 in MCF-7 cells markedly induced activation of ERK1/2 as determined with a phospho-specific antibody (Fig. 6A). ERK1/2 can stimulate cell proliferation in part by enhancing AP-1 activity resulting in cyclin D1 induction [Treinies, 1999 #4501]. Indeed, cyclin D1 levels were elevated in MCF-7/SPHK1 cells (Fig. 6A). Moreover, the specific ERK inhibitors UO126 and PD098059 eliminated the growth advantage attributed to the expression of SPHK1 in a dose-dependent manner (Fig. 6B), further confirming the importance of ERK activation. In contrast, the effect of SPHK1 was independent of the PI3 kinase pathway. First, expression of SPHK1 had no effect on Akt activation as determined by immunoblotting with a phospho-specific Akt antibody (Fig. 6A). Secondly, LY294002, a specific PI3K inhibitor did not significantly inhibit the mitogenic effect of SPHK1 (data not shown). In addition the proliferative effect of SPHK1 was independent of its anti-apoptotic function, since the proliferation of MCF-7/SPHK1 cells was not affected by the pan caspase inhibitor zVAD-fmk (data not shown). Furthermore, no differences were observed in Bax or phosphorylated JNK in proliferating cells (Fig. 6A), suggesting that SPHK1 utilizes different intracellular signals to regulate proliferation and survival in MCF-7 cells.

SPHK1 Promotes Tumorigenesis of MCF-7 Cells in Nude Mice

Because SPHK1 enhances survival, stimulates growth, and induces anchorage-independent growth of MCF-7 cells, it was of interest to determine its effect on tumor formation. MCF-7/SPHK1 cells injected subcutaneously into the mammary fat pads of ovariectomized nude mice formed larger and more tumors than MCF-7/vector cells in mice implanted with estrogen pellets (Fig. 7). In agreement with the estrogen-dependent nature of MCF-7 cell growth, no measurable tumors were formed in ovariectomized mice lacking estrogen pellets (data not

shown). MCF-7/SPHK1 tumors had a statistically significant larger mean volume. As MCF-7/SPHK1 and MCF-7/vector cells were injected in contralateral sides of each mice to avoid inter-animal variability, the difference in tumor volumes between MCF-7/SPHK1 and MCF-7/vector for each animal can be used to determine global tumor grow rates which were significantly higher in SPHK1 expressing cells (SAS PROC GLM $p=0.001$). Continuous transgene expression was confirmed by SPHK assays in tumors harvested 8-weeks after inoculation. Tumors derived from MCF-7/SPHK1 cells had 21 to 24-fold higher SPHK activity than those derived from MCF-7/vector cells. This difference in activity is comparable to that measured in the original cell lines (16 to 20-fold), indicating stable SPHK1 expression in the tumors.

The histopathologic characteristics of size-matched tumors, harvested 4 or 8 weeks after inoculation, were compatible with breast adenocarcinoma and did not include differential features in malignancy grade or apoptotic index as determined by hematoxylin-eosin staining (H-E) and DNA end-labeling (TUNEL), respectively (Fig. 8). However, higher microvessel density was found in the periphery of tumors overexpressing SPHK1 in sections stained both with H-E and by immunohistochemistry of coagulation factor VIII (Fig. 8). In agreement, an increase in edge-associated microvessels was previously found to be associated with optimal growth of tumors derived from MCF-7 cells [McLeskey, 1998 #4493].

DISCUSSION

The development of neoplasia results from cell accumulation due to enhanced proliferation, decreased cell death or a combination of both processes. Tumor growth is ensured by the acquisition of several oncogenic traits: self sufficiency in growth signals; insensitivity to anti-growth signals; evasion of apoptosis; sustained angiogenesis; limitless replicative potential; and tumor invasion and metastasis. S1P, the product of SPHK, may be involved in oncogenesis since it may contribute to three of these traits depending on the cell type involved. First, elevated S1P levels promote cell proliferation in non-transformed cells in the absence of growth factors and it has been suggested that SPHK is an oncogene, acting in a Ras-dependent signaling pathway (Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.). Second, elevating S1P levels by either exogenous addition or by SPHK overexpression, acts upstream of caspase activation to inhibit apoptosis induced by a variety of stimuli (Edsall, et al., 2001 *J. Neurochem.* **76**, 1573-1584.; Olivera, et al., 1999 *J. Cell Biol.* **147**, 545-558.). A recent study demonstrates that S1P prevents

apoptosis and executioner caspase-3 activation by inhibiting translocation of cytochrome c and Smac/DIABLO from mitochondria to the cytosol in human acute leukemia cells. This suggests that SPHK, inhibits the apoptotic cascade upstream of the release of these mitochondrial apoptogenic factors [Cuvillier, 2001 #4473]. Finally, S1P may promote angiogenesis as suggested by formation of endothelial tubes ex vivo (English, et al., 2000 *FASEB J.* **14**, 2255-2265; Wang, et al., 1999 *J. Biol. Chem.* **274**, 35343-35350.), by enhancement of mature neovessels in vivo [Lee, 1999 #3903; Lee, 1999 #3892; Garcia, 2001 #4413], and by defective maturation of blood vessels in mice with a deletion of the S1P receptor Edg-1 [Liu, 2000 #4418]. Furthermore, DMS, a specific SPHK inhibitor, has cytostatic effects on gastric tumors *in vivo* (Endo *et al.*, 1991 *Cancer Res.* **51**, 1613-1618.). Therefore, it is important to characterize the role of S1P derived from SPHK in the development of cancer.

Increased resistance to apoptosis is a hallmark of most types of cancer. MCF-7 cells have a deletion of caspase-3 and utilize caspase-7 to execute programmed cell death [Cuvillier, 2001 #4340]. Our results support a role for SPHK in regulation of cell survival to promote tumor growth. Inhibition of caspase-7 activation and PARP cleavage, correlated with enhanced survival of MCF-7/SPHK1 cells after treatment with doxorubicin, TNF- α , sphingosine or DMS. Our results suggest that SPHK1 enhances the resistance of MCF-7 cells to apoptotic stimuli by curtailing Bax, and reducing JNK activation. In agreement, the anti-apoptotic effect of S1P has been correlated with downregulation of Bax expression in Jurkat cells (Goetzl, et al., 1999 *J. Immunol.* **162**, 2049-2056.) and inhibition of phosphorylation of JNK in PC12 cells (Edsall, et al., 2001 *J. Neurochem.* **76**, 1573-1584.).

It seems most likely that the effects of SPHK1/S1P on survival are mediated through intracellular actions because in many cell types, dihydroS1P which binds and activates all of the Edg-1 family, does not protect from apoptosis [Van Brocklyn, 1998 #2303; Olivera, 1999 #3878; Edsall, 2001 #4145; Morita, 2000 #4165; Rosenfeldt, 2001 #4380]. However, compelling evidence indicates that SPHK1/S1P is able to promote cell growth not only by intracellular (reviewed in (Pyne and Pyne, 2000 *Biochem. J.* **349**, 385-402.; Spiegel and Milstien, 2000 *Biochim. Biophys. Acta* **1484**, 107-116.) but also by extracellular actions (reviewed in [Goetzl, 2001 #4251; Hla, 2001 #4476]. A clear differentiation of these actions may be confounded by the recent report that S1P activates sphingosine kinase and stimulates its own production after Edg receptor engagement [Meyer zu Heringdorf, 2001 #4386]. Conversely, intracellularly

generated S1P is able to stimulate Edg receptors in an autocrine or paracrine fashion, even when secretion is undetectable by mass level analysis (Hobson *et al.*, 2001 *Science* **291**, 1800-1803.). In contrast, elevated S1P concentrations have been detected in ascitic fluid from ovarian cancer patients [Hong, 1999 #4464]. However, several lines of evidence suggest that SPHK1-promoted proliferation of MCF-7 cells is independent of binding of S1P to its receptors. First, neither S1P or dihydroS1P stimulate growth of parental MCF-7 cells. Second, SPHK1/MCF-7 cells do not release detectable amounts of S1P into the medium. Third, whereas previously it has been suggested that proliferation of MCF-7 cells induced by activation of S1PRs is due to enhanced secretion of insulin-like growth factor (IGF-II) and activation of IGF-IR (Goetzl, et al., 1999 *Cancer Res.* **59**, 4732-4737.), known to induce Akt-dependent mitogenesis [Butler, 1998 #4503], anti-IGF-IR did not block proliferation induced by SPHK1 and its expression did not increase phosphorylation of Akt. However, it still remains possible that spatial and temporal generation of S1P in the vicinity of its receptors may activate ERK leading to proliferation. The proliferative advantage attributable to SPHK1 together with its anti-apoptotic function, may favor tumor progression. Indeed, MCF-7 cells overexpressing SPHK1 exhibit robust anchorage-independent growth and result in improved tumorigenesis in nude mice.

Estrogens strongly promote proliferation of mammary cells after binding to ER. Malignant progression of breast cancer correlates with acquisition of estrogen-independent growth by mechanisms that are not fully understood [Lippman, 2001 #4494]. It is thus important to understand signaling cooperation in estrogen-dependent breast cancer cells, such as MCF-7 cells, in order to identify cross-talk pathways that could impinge on cancer progression. Interestingly, SPHK1 enhances the growth effects of estradiol, and ICI inhibits the mitogenic effects of SPHK1. However, SPHK1 does not affect ER levels or regulation of estrogen responsive genes, and conversely, estradiol has no effect on SPHK1 activity, suggesting a that S1P may act independently. ERK activation is dispensable for estrogen-induced mitogenesis [Caristi, 2001 #4502] but may be essential for S1P mitogenesis as ERK suppress the proliferative effect of SPHK1 in MCF-7 cells. Taken together, these results suggests a mechanism whereby S1P promotes estrogen-dependent tumor progression. Interestingly, constitutively active Ras mutations, which stimulate ERK, also activate SPHK1 (Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.), raising the possibility of a positive feedback loop involving ERK that ensures optimal cell growth. It is noteworthy, that SPHK1 stimulates the growth of MCF-7 cells cultured in full

serum. These suggests that in addition to a role initiating oncogenic transformation (Xia, et al., 2000 *Curr. Biol.* **10**, 1527-1530.), S1P could also promote the growth of established tumors.

Angiogenesis, the formation of new blood vessels, is an essential requirement for tumor growth. S1P promotes vessel formation through Edg-1 mediated mechanisms that are sensitive to pertussis toxin and C3 exoenzyme (English, et al., 2000 *FASEB J.* **14**, 2255-2265; Lee, et al., 1999 *Cell* **99**, 301-312; Lee, et al., 1999 *Biochem. Biophys. Res. Commun.* **264**, 743-750; Wang, et al., 1999 *J. Biol. Chem.* **274**, 35343-35350.). Disruption of the Edg-1 gene in the mouse results in embryonic lethality due to a vascular maturation defect [Liu, 2000 #4418]. Moreover, it has been suggested that synergism between Edg-1, Edg-3 and various angiogenic factors such as vascular endothelial cell growth factor (VEGF) and fibroblast growth factor (FGF) is necessary for mature neovessel formation in vivo in the presence of S1P (Lee, et al., 1999 *Cell* **99**, 301-312.). In agreement we detected an increase in edge-associated microvessels in MCF-7 tumors overexpressing SPHK1. Dramatically enhanced neovascularization by SPHK1 adenovirus or SPHK1 transduced AE1-2a cells implanted into the Matrigel plugs containing FGF-2 [Ancellin, 2001 #4504]. In contrast, the effect of SPHK1 overexpression on new blood vessel formation in MCF-7 derived tumors was much smaller. This could be due to differences in cell type although in both cases, there was no detectable secretion of S1P. Thus, although we cannot exclude a paracrine contribution of S1P on endothelial cells invading the tumors, this is likely not the major factor contributing to increased numbers and larger tumors produced by MCF-7/SPHK1 cells. Collectively, our results suggest that SPHK1 may play an important role in breast cancer progression by regulating tumor cell growth and survival. If so, SPHK inhibition might be a useful adjunct to anti-estrogen treatment of breast cancer.

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TABLE I. CELL CYCLE ANALYSIS OF STABLY TRANSFECTED MCF-7 CELLS.

Cells	G₀/G₁ (%)	S (%)	G₂/M (%)
SPHK1	53.7 ± 0.6*	27.5 ± 0.1	18.9 ± 0.5*
vector	59.8 ± 0.4	25.0 ± 0.3	15.2 ± 0.8

Cells were harvested during exponential growth 48 h after seeding in media containing 10 % FBS. Cell cycle distribution was determined by flow cytometry as described in Materials and Methods. Asterisks indicate statistical significance by Student-t test ($P < 0.05$). Results are representative of two independent experiments in duplicates expressed as means \pm S.E.

TABLE II. EXPRESSION OF THE ESTROGEN RECEPTOR ON THE STABLY TRANSFECTED MCF-7 CELLS.

Cells	E2 Receptor (fmol/mg)
SPHK1	22.45 ± 5.39
Vector	15.48 ± 2.47

After thorough washing with serum-free phenol red-free media, cells were maintained in 5 % CCS media for 48 h prior to harvesting. Estrogen receptors were quantitated by immunoassay as described in Materials and Methods. Results are means ± standard error of six independent experiments.

FIGURE LEGENDS

Figure 1. SPHK1 overexpression enhances survival of MCF-7 cells to stress stimuli. Subconfluent MCF-7 cells stably transfected with vector or SPHK1 were cultured in serum-free media in the presence of (A) 1 μ g/ml doxorubicin, (B) 100 ng/ml tumor necrosis factor- α (TNF- α) plus 0.5 μ g/ml cycloheximide (CHX), (C) sphingosine or (D) N,N-dimethylsphingosine (DMS) as indicated. Cell viability was determined by the MTT assay. Data are the means \pm S.E. of triplicate determinations. Similar results were obtained in at least three independent experiments. (E) There was no reduction in viability of either vector or SPHK1 transfectants even after 72 h in serum-free medium (SF) compared to 10% serum (FBS).

Figure 2. SPHK1 overexpression reduces caspase-7 activation. Vector and SPHK1 transfected MCF-7 cells cultured in serum-free media were treated for 48 h without (-) or with (+) 1 μ g/ml doxorubicin (DOXO) or 10 μ M N,N-dimethylsphingosine (DMS), and equal amounts of cell lysates were analyzed by immunoblotting using antibodies against PARP and caspase-7. Arrowheads indicate full-length PARP. Migration of the cleaved fragments of caspase-7 (p20) and PARP (p89) are indicated. Extracts of LNCaP cells treated with okadaic acid (OA) serve as positive control. Similar results were obtained in three independent experiments.

Figure 3. Expression of SPHK1 in MCF-7 cells decreases phosphorylated JNK after serum withdrawal. Vector and SPHK1 transfected MCF-7 cells cultured in serum-free media were treated for 48 h without (-) or with (+) 1 μ g/ml doxorubicin (DOXO) and cell lysates analyzed by immunoblotting with anti-BAX. After stripping, the blot was probed with anti-phospho-JNK and anti-JNK antibodies. Similar results were obtained in three independent experiments.

Figure 4. Effect of SPHK1 expression on growth of MCF-7 cells. Vector and SPHK1 transfected MCF-7 cells were plated at low density, and cultured in (A) 0.5 %, (B) 10 % FBS in the presence of the indicated concentrations of DMS (C) Growth in soft-agar was determined after 7 and 10 days as described in Materials and Methods. Results are representative of two to five independent experiments performed in triplicate. Bars indicate standard errors.

Figure 5. Enhancement of MCF-7 cell growth by enforced expression of SPHK1 is estrogen dependent. (A) MCF-7 cells were plated at low density and grown in 5 % CCS. At the indicated times cell growth was measured by the crystal violet assay. Vehicle or S1P (100

nM) or dihydro-S1P (100nM) were replenished every two days in phenol red-free media containing 5 % CCS. (B) Vector and SPHK1 transfected MCF-7 cells were plated at low density and grown in 5 % CCS or 10% FBS and cell growth was measured . (Peyton please add)) (C) 17- β -estradiol (E2, 100 nM) and (D) the anti-estrogen ICI 182,780 (ICI, 1 nM) were replenished every two days in phenol red-free media containing 5 % CCS. Results are representative of two independent experiments performed in quadruplicate. Bars indicate standard errors.

Figure 6. Expression of SPHK1 in MCF-7 cells stimulates ERK. Vector and SPHK1 transfected MCF-7 cells were cultured in 10% FBS. Cytosolic fractions were prepared and equal amounts of protein were analyzed by Western analysis using antibodies specific for phospho-ERK and then stripped and reprobed with anti-p42/p44 MAPK (ERK); cyclin D1; phospho-Akt and then stripped and reprobed with anti-Akt; BAX; phospho-JNK and then stripped and reprobed with JNK antibodies. Similar results were obtained in three independent experiments. P- indicates phosphorylated proteins. **(B) Enhancement of MCF-7 cell growth by enforced expression of SPHK1 is dependent on ERK activation.** Cells were plated and grown in 10 % FBS the ERK inhibitors UO126 and PD98059 (PD) were replenished at the indicated concentration every two days. Cell growth was determined with crystal violet after 8 days. Bars indicate standard errors of an experiment performed in quadruplicate.

Figure 7. Enforced expression of SPHK1 in MCF-7 cells promotes tumorigenesis in nude mice. (A) Vector and SPHK1 transfected MCF-7 cells (5×10^6) were inoculated subcutaneously in opposite sides of the abdominal wall of ovariectomized nude mice implanted with 17- β estradiol pellets. Tumor size was measured on the indicated days. Results are representative of two independent experiments with n=5 and n=20. The differences in tumor size produced by vector and SPHK1 transfected cells was statistically significant (Wilcoxon signed rank test, $P < 0.05$) at the indicated days. **(B)** An example of tumor size in a mouse sacrificed after 8 weeks.

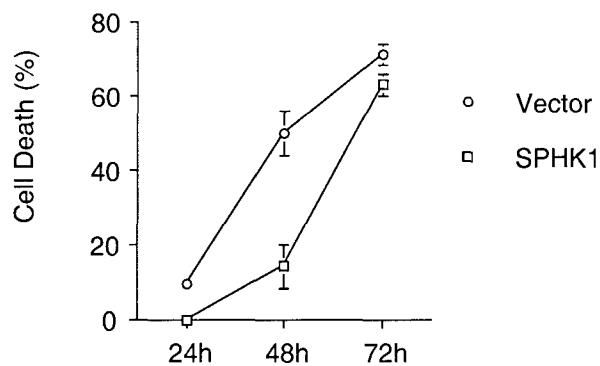
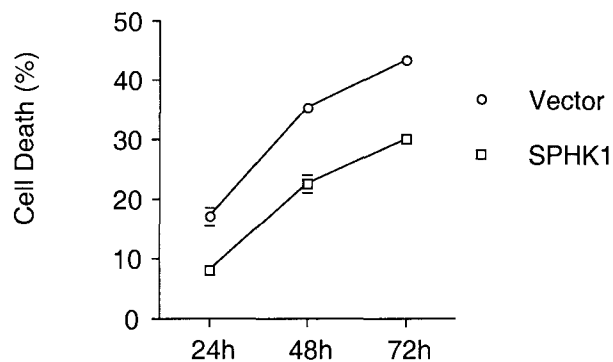
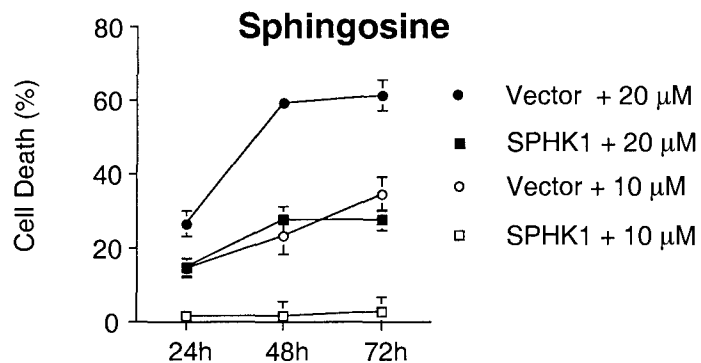
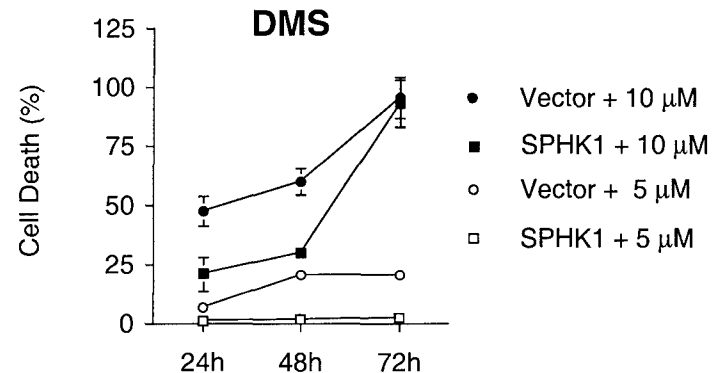
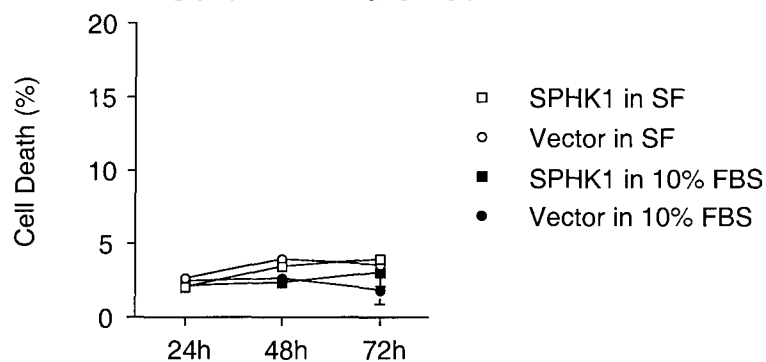
Figure 8. Effect of SPHK1 on tumor vascularization. (A) Tumor edge-associated microvessel quantification was performed on hematoxylin-eosin (H-E) stained sections, counting erythrocyte containing vessels in areas of high density in three independent tumors for each group. Bars indicate standard errors of 4 to 8 counts. Asterisk indicates statistical significance using Student-t test ($p \leq 0.03$). (B) A representative tumor section is shown. Adjacent sections were stained

with H-E or with anti-Von Willebrand factor (V-W). Brown color and arrowheads indicate endothelial cells and representative blood vessels, respectively.

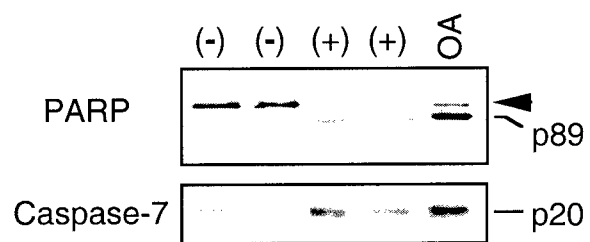
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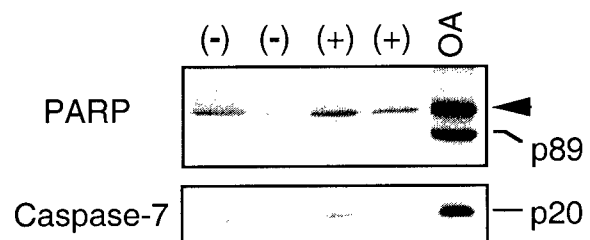
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A**Doxorubicin****B****TNF- α + CHX****C****Sphingosine****D****DMS****E****Serum Withdrawal**

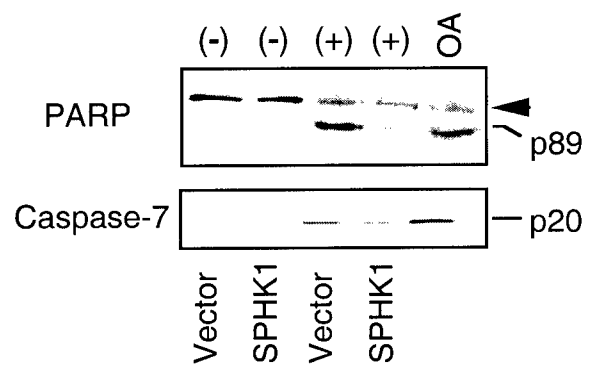
DOXO



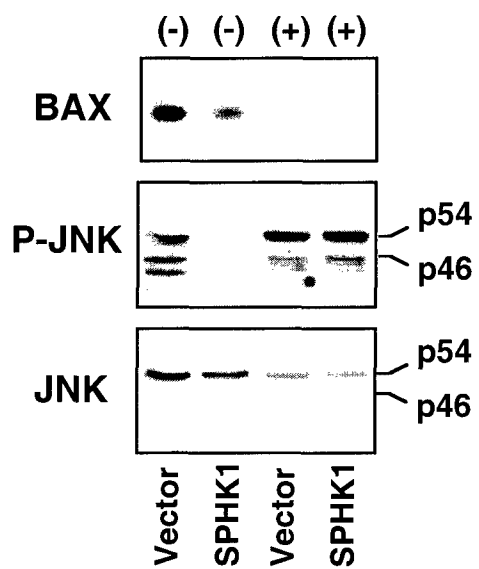
Sph

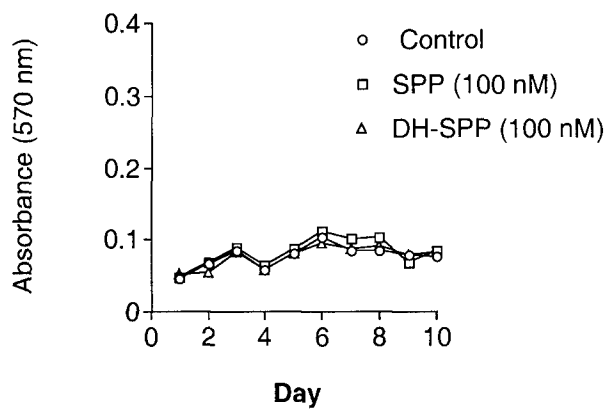
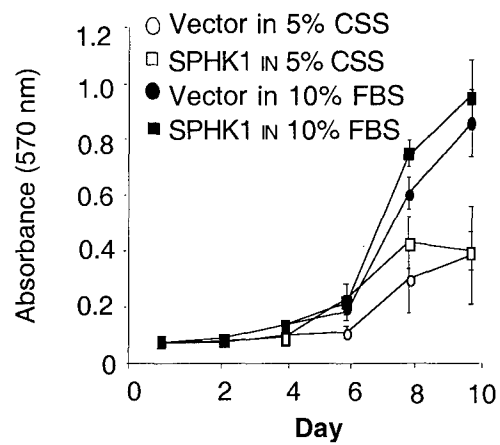
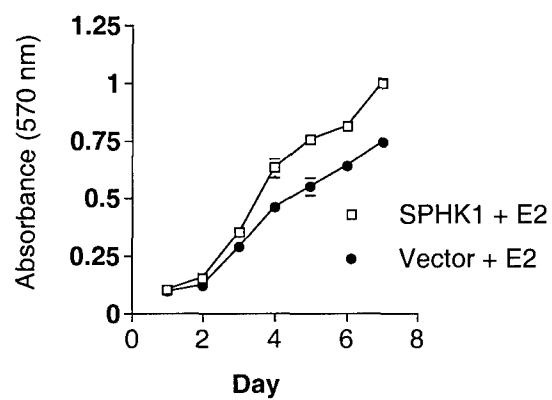
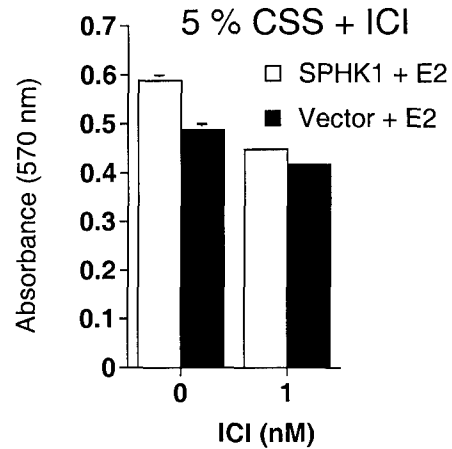


DMS

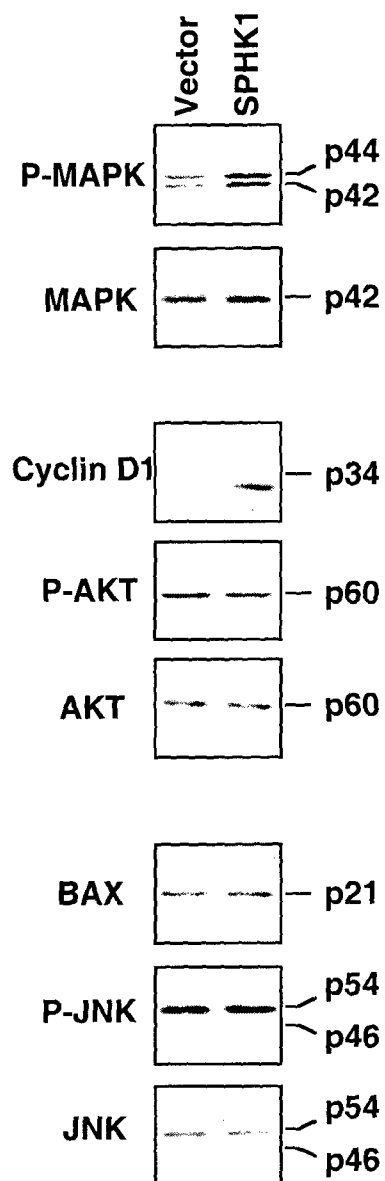


DOXO

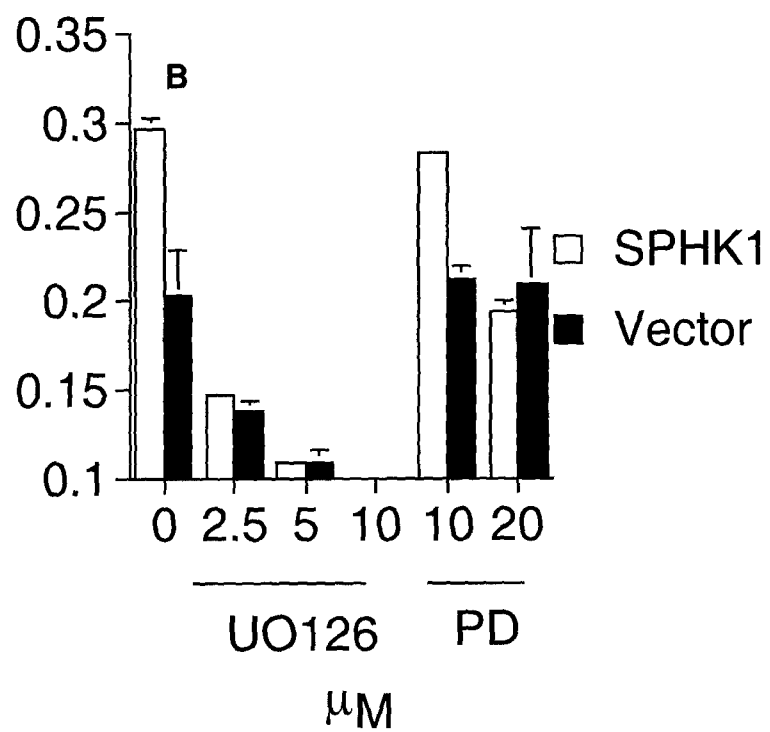


A**5 % CSS****B****C****5 % CSS****D****5 % CSS + ICI**

A



Absorbance (570 nm)



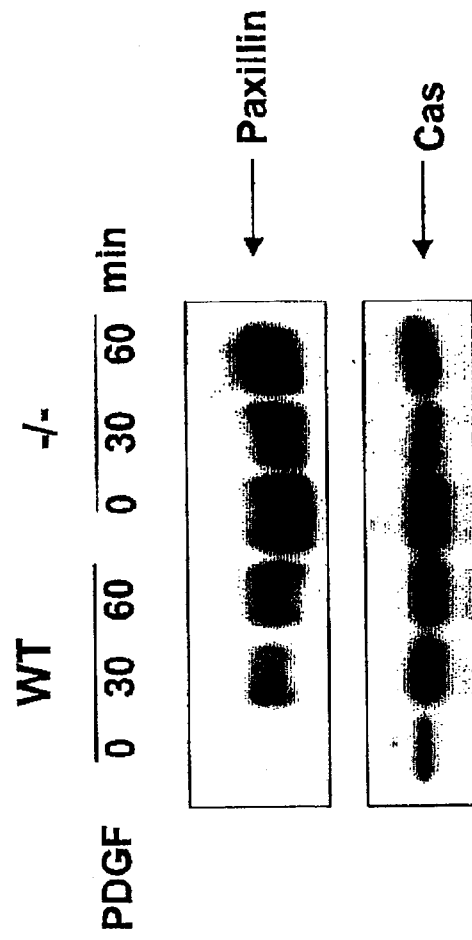
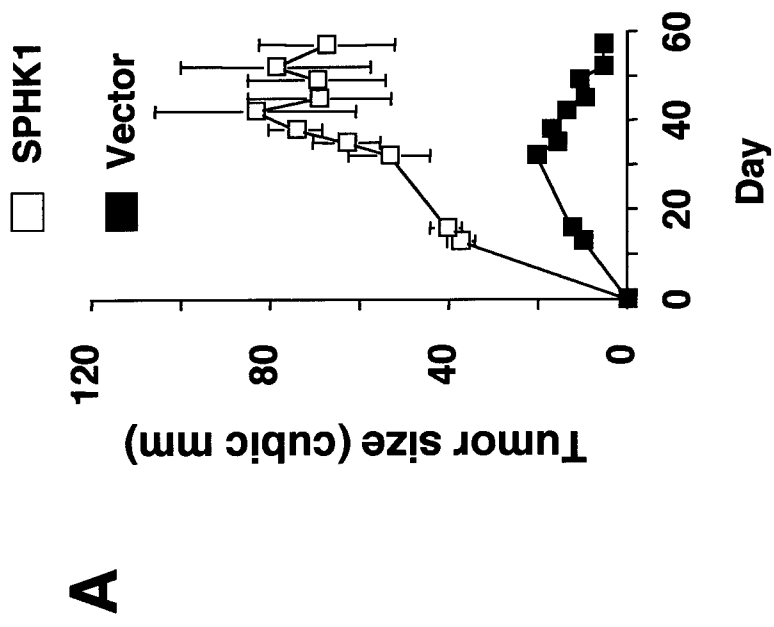
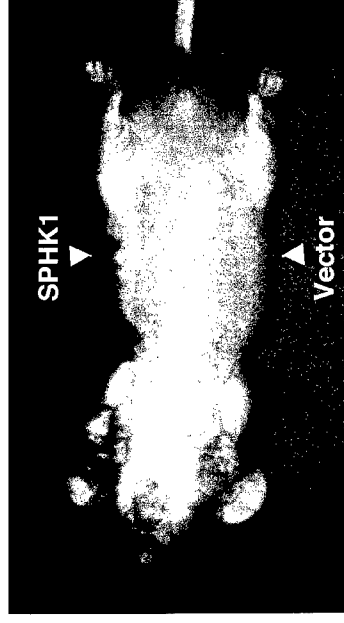


Figure 6

Figure 7



B



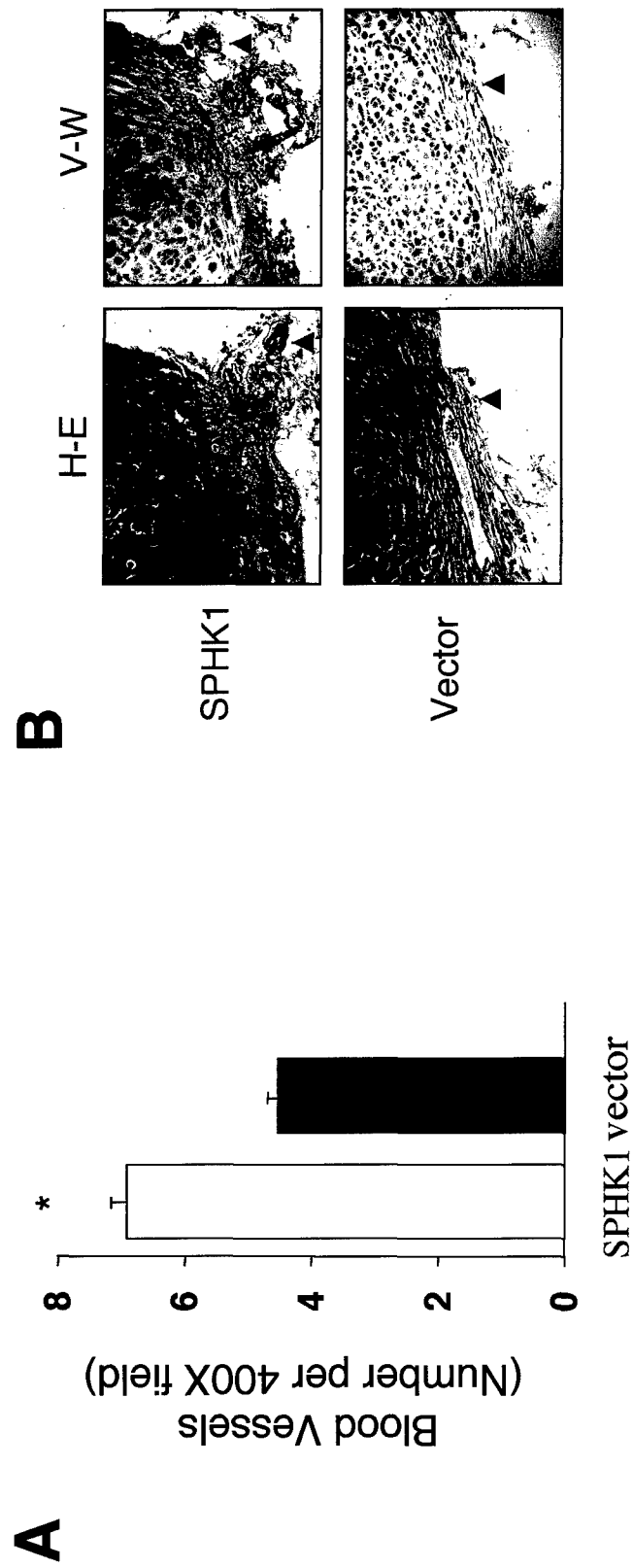


Figure 8



REPLY TO
ATTENTION OF

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

MCMR-RMI-S (70-1y)

15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
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